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Absence of Endotoxic Activity in Materials Derived Without Bacterial Contamination from Mammalian Tissue. (26134)

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Much literature has accumulated on the biologic effects of endotoxins, which are recognized as deriving mainly, if not exclusively, from Gram-negative bacteria. There are, however, a number of reports on endotoxic properties of materials extracted from mammalian tissues. Atkins(1) has summarized these contributions. In addition, Antopol and coworkers(2-4) found that extracts of normal and malignant tissues were capable of eliciting several characteristic endotoxic reactions, *viz.*, the Shwartzman phenomena in rabbits and tumor damage in mice; more recently, Martin and Spielmann obtained(5) materials with pyrogenic potency from human erythrocytes.

Earlier experiments(6) in this laboratory had yielded preparations, from mammalian tissues, which exhibited 3 endotoxic properties. Some of these preparations were later found(7) to induce the entire array of host reactions as do bacterial endotoxins.

To obtain a reproducible methodology, the procedures first employed were subjected to systematic study in which the effects of variations in technic were examined. This communication reports that with some procedures the final preparations were devoid of biologic activity; when the products displayed endotoxic potency, it was found that the variations in technic had permitted bacterial growth.

Materials and methods. Various tissues (whole blood, red cells, red cell stroma, and tumor) from several mammalian species (mouse, rabbit, and man), after immersion in an aqueous medium containing phenol and trichloroacetic acid, were treated as outlined previously(8). Other precautions, taken to preclude bacterial growth during protracted chemical manipulations, included: buffer solutions were freshly prepared and autoclaved; the trypsin (pancreatin), after purification to remove polysaccharides, was sterile, and negative in the tumor assay even at the high dose of 20 mg per mouse; additional phenol was employed during dialysis after treatment with trypsin; and pyrogen-free water was employed throughout.

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Among the variations in technic examined for their influence upon the properties of the final product was omission of layering with toluene during treatment with trypsin.

Small aliquots of all solutions, and of the materials at all stages of the manipulations, were examined for presence of bacteria by inoculation into thioglycollate fluid medium or semi-solid medium and by subsequent plating on nutrient, EMB or blood agar. When bacterial growth was evident, the organisms were isolated and their reaction to the Gram stain determined.

Elicitation of hemorrhagic necrosis(9) in sarcoma 37 was the biological activity measured in the bioassays.

Results. In experiments with red cell stroma, the product obtained when toluene had been employed was found to be inactive; a parallel experiment, in which toluene had been omitted, yielded a product active in the tumor assay. Culture of the tryptic digest was negative in the former instance; bacterial contamination with Gram-negative species was evident in the latter.

The lack of activity of this non-contaminated preparation from stroma raised the question as to whether contamination inadvertently occurred with other preparations despite the precautions taken. Accordingly, systematic bacteriologic examination was instituted at the various stages of the processing of materials. It was found that all steps in the protracted manipulations could be carried out, repeatedly, with exclusion of bacteria in the open chemical laboratory. However, the end products, when obtained under this kind of bacteriologic control, displayed no endotoxic activity as assessed in the tumor assay.

The products we prepared from human red cell stroma by Martin's method(5) also were inactive in the tumor assay even when as large a dose as 5 mg per mouse was employed; a low order of pyrogenicity was exhibited by this product which had a M.P.D. of 150 μg.

The negative products obtained under satisfactory bacteriologic controls were high in nitrogen, yielding values commonly found in mucoproteins.

Discussion. Inasmuch as this kind of bacteriologic data is not available on the earlier preparations(6), of which some may have been contaminated, the significance of their endotoxic properties is now reassessed. It was stated(7): that the micro-organisms which plants and animals ordinarily contain might be the source of activity of the polysaccharide complexes obtained from their tissues; and that pyrogenic bacteria, inadvertently introduced during handling of the source materials, were to be reckoned with in view of their ubiquity, high potency, and relative ease of contamination by them. However, because of the considerations stated, these factors did not appear to account for the results obtained. Results(7) reported for the earlier preparations are now viewed as possibly having been due, in an undeterminable number of instances, to inadvertent introduction of bacteria.

Nevertheless, it would be premature to conclude that tissues do not contain components with endotoxic properties. For one thing, Bennett has, under strictly controlled conditions, obtained pyrogen from turpentine-induced exudates(10) in a critical extension of Menkin's(11) finding, and also from polymorphonuclear leucocytes(12). We have speculated(7) that, if tissues contain endotoxic polysaccharides, they would not exhibit their characteristic properties in their usual state of combination with protein but only after they were separated from the complex. The high nitrogen values obtained under current conditions of our experiments indicate that the enzymes employed apparently did not split off the protein moiety with consequent liberation of polysaccharide. This experience is consistent with those of others who have also encountered resistance of mammalian mucoproteins to proteolytic enzymes.

Whether the polysaccharides in these inactive complexes would, or would not, exhibit endotoxic behavior when success is achieved in liberating them from combination is not known. This is being investigated.

Summary. In the attempted isolation from mammalian tissues of polysaccharides with endotoxic properties, only materials devoid of such activity were obtained under conditions in which exclusion of bacteria was demonstrated.

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Effect of Nitrogen Mustard in Rabbits Following Exposure to X-Irradiation.

(26135)

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Bone marrow depression represents one of the problems encountered in the use of chemotherapeutic agents in treatment of malignant disease. At this Institution we have treated several patients with large doses of nitrogen mustard (0.95-1.8 mg/kg) for Hodgkin's disease(1). Results of such therapy suggest that patients who have received prior x-irradiation have a more severe and prolonged bone marrow depression than those not previously exposed. Because of the small number of patients and variation in the clinical setting, definite conclusions concerning this point have been difficult to reach. This report deals with the results of a comparable study done in experimental animals in an attempt to determine whether or not there is a difference in response between irradiated and non-irradiated animals following administration of nitrogen mustard.

Methods. Ten adult, female rabbits used in the study were divided into 2 groups of 5 each. One group was given x-irradiation while the other served as control. Two courses of x-ray were given, 250 r whole body irradiation over a 10-minute period. Following each course of exposure, a suitable period of 5-6 weeks was allowed for recovery of bone marrow as manifested by return of normal peripheral blood counts. Five weeks following the last dose of x-ray, both groups of animals were given 1.5 mg/kg nitrogen mustard intravenously. Peripheral blood counts, including red blood cell count, hemoglobin, hematocrit, white blood cell count and platelet counts (direct), were performed prior to start of the study, at weekly intervals during experiment and one week following administration of nitrogen mustard. All blood specimens were obtained by cardiac puncture without difficulty. Bone marrow aspiration was performed prior to x-ray exposure and one week following nitrogen mustard administration in the surviving animals.

Results. In the control group one animal sustained a cutaneous injury which became

TABLE I. Hematologic Response of Rabbits Following Exposure to X-Irradiation and Administration of Nitrogen Mustard.

		Control group				Irradiated group				
		1	2	3	4	1	2	3	4	5
Red cell count $\times 10^3$	a	4.2	5.2	4.4	4.9	5.4	4.0	4.9	3.5	4.6
	b	3.4	4.2	4.2	4.6	4.4	4.8	5.2	3.4	4.4
	c	3.9	4.7	4.4	4.6	4.5	4.6	4.7	4.0	4.6
	d	3.7	4.4	4.3	4.9	4.3	3.3	—	3.6	—
Hematocrit	a	26.5	32.5	28.0	30.5	33.0	28.0	32.0	26.0	33.5
	b	34.5	37.5	35.0	40.0	40.0	34.5	37.0	27.5	32.0
	c	34.0	39.0	35.0	37.0	36.0	34.5	36.5	28.5	35.0
	d	33.0	34.0	33.0	29.0	34.5	30.5	—	28.5	—
Hemoglobin, g %	a	9.1	10.6	9.4	11.5	11.9	10.3	10.5	9.6	11.9
	b	10.5	11.4	11.0	12.5	12.1	10.1	11.1	8.8	9.6
	c	10.1	12.1	10.5	11.1	11.3	10.0	11.0	8.3	10.0
	d	10.3	11.2	10.1	9.7	11.4	10.1	—	8.3	—
White cell count $\times 10^3$	a	10.1	9.4	9.5	9.0	8.6	9.8	5.0	11.1	6.7
	b	8.2	10.0	7.9	5.3	3.3	2.2	3.5	3.7	2.4
	c	9.7	12.8	1.7	15.0	5.7	8.9	10.0	13.0	13.0
	d	11.4	8.6	9.5	6.7	4.3	4.7	—	1.4	—
Platelet count $\times 10^3$	a	200	415	280	377	483	530	390	315	330
	b	445	340	360	450	210	100	205	40	130
	c	315	345	300	415	295	385	275	315	440
	d	547	537	255	415	147	450	—	few on smear	—

a = control peripheral blood counts.
b = maximum peripheral count depression following second x-irradiation exposure.
c = peripheral blood counts prior to administration of nitrogen mustard.
d = " " " one wk following nitrogen mustard.

secondarily infected, was resistant to therapy and had to be sacrificed. The remainder of the animals in this group tolerated the nitrogen mustard well and showed no significant depression of the peripheral blood count. Bone marrow specimens were diluted with peripheral blood but showed normal activity of all cell types. They remained well throughout the study, and there were no deaths following nitrogen mustard.

There were 3 deaths among the 5 animals of the irradiated group. One of these showed evidence of generalized debility with weight loss and lethargy during the recovery period following x-irradiation despite return of the peripheral blood count to normal levels. On the day following injection of nitrogen mustard, the animal was found dead in its cage. At autopsy no specific organ abnormality was noted. The second animal which died showed lethargy 4 days post-injection. A blood count at this time showed a marked neutropenia and a marked depression of platelets on the peripheral smear. Marrow aspirate showed depression of granulocytic and megakaryocytic activity. The animal died on the following day and autopsy revealed massive hemopericardium and hemorrhage into the left lung, which was felt was due to the trauma of cardiac puncture and associated thrombocytopenia. The third animal showed no immediate ill effects of nitrogen mustard therapy, but was found dead in its cage 4 days post-injection. Autopsy revealed no gross cause of death. Hematologic data are shown in Table I.

Discussion. Our experience with administration of large doses of nitrogen mustard to patients with Hodgkin's disease had suggested that previous treatment with x-irradiation, even in those with apparent recovery of normal marrow function, caused severe and prolonged post-mustard marrow depression. Mrazek and Wachowski(2) have also suggested that previous treatment with irradiation or chemicals was the factor most directly related to the occurrence of peripheral blood depression in patients who received doses of nitrogen mustard. The Council on Pharmacy and Chemistry of the American Med. Assn.(3) has stated that "a severe and even uncontrollable depression of the hemopoietic system may follow the usual dose of nitrogen mustard, particularly in patients with widespread disease and in those patients who have had previous treatment with other similar agents or x-ray."

In an attempt to clarify the problem of this effect of previous exposure to x-ray and subsequent response to nitrogen mustard, a similar experimental study was devised utilizing the rabbit as the experimental animal. There was a marked difference in response of the two groups. In the control group which received no previous x-ray, but did receive 1.5 mg/kg nitrogen mustard, there were no deaths and no depression of hemopoietic activity. In the x-irradiated group, with all animals showing normal hemopoietic activity prior to receiving nitrogen mustard, 3 of 5 animals died following injection. In 2 of these, the cause of death was not demonstrated at autopsy. Blood counts were not obtained prior to death. In the third, bone marrow and peripheral blood count revealed marked depression of hemopoietic activity and death secondary to massive hemopericardium. That this complication was, in part, due to thrombocytopenia is suggested by the fact that no evidence of bleeding due to cardiac puncture in the control group was noted.

Summary. Rabbits which had been exposed to whole body x-irradiation showed a significant mortality rate following injection of 1.5 mg/kg body weight nitrogen mustard in contrast to the control group where no deaths occurred. This difference was noted even though bone marrows of the x-irradiated group were functionally normal when nitrogen mustard was administered.

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Effects of Brucellaphage on Extracellular and Intracellular *Brucella abortus*.* (26136)

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During studies on the activity of a brucellaphage(1) capable of lysing smooth *Brucella abortus* it was observed that UV-inactivated phage preparations retained their ability to inhibit multiplication of *Br. abortus* organisms. Similar findings have been reported for *Escherichia coli*(2). Efforts now have been made to ascertain whether these inhibitory effects were caused by the protein moiety of the phage, and also whether such effects could be exerted intracellularly as well as extracellularly. An opportunity to examine this latter aspect was afforded by concurrent studies on intracellular multiplication of *Br. abortus* within monocytes maintained *in vitro*(3-5).

Methods. Smooth organisms of *Br. abortus*, strain SA-S(4), were maintained and grown using tryptose agar, buffered beef extract broth (BBE), or, as described below, within monocytes. Brucellaphage(1) was propagated by mixing a BBE suspension of 24-hour-old *Br. abortus* organisms with phage in the ratio of 1:5, allowing adsorption for 30 mintues at 37°C, and plating the mixture upon tryptose agar. Twenty-four hours later the surface of the agar was washed with saline, a few ml of chloroform was added to the washings to kill all remaining viable brucellae, and the mixture was allowed to stand overnight at 4°C. Phage plus bacterial debris were then sedimented by centrifugation,

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and the phage was eluted from the sediment into phosphate buffer at pH 7.0. Successive elutions and centrifugation (1 to 5), yielded phage preparations having a titer of about 1 x 10^{10} plaque-forming units (pfu) per ml. Phage was titrated by plaque count; serial dilutions being placed onto respread, young (8-hour-old) surface cultures of susceptible *Br. abortus*. UV-inactivation (99%) of phage was achieved with a "Universal Illuminator" (40 cm, 2 minutes), heat-inactivation (99.99%) by heating to 70°C for 10 minutes.

Cultures of guinea-pig monocytes were prepared according to the methods of Pomales-Lebrón and Stinebring(3), except that monocytes were allowed to ingest brucellae while both were in suspension at 37°C for 1 hour. Following such periods of ingestion, the monocytes were washed free from extracellular brucellae and resuspended in serum-Hanks' medium containing streptomycin (10 γ per ml). One-ml portions of this brucella-containing monocyte suspension were then mixed with either phage or buffer (the latter served as control) and introduced into Porter flasks. Thus, in any given experiment, phage-exposed and unexposed monocytes were identical in regard to prior history and brucella content. After 2 hours incubation at 37°C, when the monocytes had become attached to the floor of the flask, the supernatant medium was replaced by streptomycin-containing serum-Hanks' medium free of phage. After desired periods of time, number of intracellular brucellae was estimated by viable counts of bacteria released from broken monocytes(5), and by direct microscopic observations on stained monocytes adhering to flying coverslips that were contained within the culture flasks. In addition, number of monocytes per flask was estimated, after staining(3), by microscopic counts on 10 separate, measured areas of the coverslip.

Results. Effects of phage on extracellular brucellae. When equal volumes of suspensions of phage (5 x 10^9 pfu per ml) and smooth *Br. abortus* (1 x 10^9 per ml BBE) were mixed, incubated on a shaker at 37°C for 30 minutes, diluted, and plated upon tryptose agar, approximately 35% of the cells were killed (judged by their inability to form detectable colonies). Table I, which shows data typical of a large series, also contains data demonstrating that a comparable inhibition of bacterial growth was obtained when UV-inactivated, but not when heat-inactivated, phage preparations were employed. The possibility of ascribing the inactivating effects exerted by UV-irradiated phage to the remaining 1% plaque-forming phage is made unlikely by the observation that untreated phage preparations diluted to a comparable pfu titer failed to affect the multiplication of susceptible bacteria. Our inability to obtain more than 40 to 45% "killing" by either untreated or UV-treated phage preparations is probably due to a lack of optimal conditions for phage adsorption onto bacteria in the present experiments.

TABLE I. Survival of *Brucella abortus*, SA-S, Following Exposure to Untreated or Treated Brucellaphage.

Brucellae exposed to:	Brucellae recovered*	% inhibition
Buffer	103 ± 5.7	—
Untreated phage	66 ± 2.8	36
UV-inactivated phage	58 ± 3.1	44
Heat-inactivated "	98 ± 4.6	None

* After plating 0.02-ml aliquots of a 10^{-5} dilution of the phage-bacterium mixture. Averages from 4 separate determinations.

Effects of phage on intracellular brucellae. When the effects of untreated, UV-inactivated, and heat-inactivated phage preparations were tested with brucellae that had previously been ingested by monocytes, the results were essentially identical with those obtained in tests with extracellular brucellae; *i.e.,* untreated or UV-treated phage, even when added following the period of ingestion of brucellae by monocytes, interfered with intracellular multiplication of the bacteria. Thus, inspection of stained preparations, obtained from replicate cultures at various times following exposure to phage, revealed a slight but consistent retardation of intracellular multiplication of brucellae in cultures exposed to untreated or UV-treated phage. Typically, the number of smooth brucellae within individual monocytes increases rapidly after a lag of approximately 12 hr(4); however, cultures exposed to untreated or

TABLE II. Distribution of *Brucella abortus,* SA-S, within Individual Brucella-Containing Monocytes Cultivated *In Vitro* in Presence or Absence of Phage Preparations. Data obtained by microscopic examination of stained material.

Time of exam.[*]	Treatment of cultures	% of monocytes containing 1-6	7-10	>10 brucellae
2	Control	77 ± 6.1[†]	19 ± 3.0	4 ± 1.2
	Active phage	83 ± 7.0	13 ± 3.1	4 ± 1.4
	UV-treated phage	85 ± 7.9	10 ± 2.2	5 ± 1.2
	Heat-treated "	89 ± 6.2	7 ± 1.2	4 ± 1.1
24	Control	46 ± 4.2	36 ± 5.9	18 ± 3.5
	Active phage	63 ± 4.4	24 ± 6.8	13 ± 2.9
	UV-treated phage	65 ± 4.5	28 ± 6.1	7 ± 2.0
	Heat-treated "	48 ± 3.9	36 ± 5.7	16 ± 3.1

[*] In hr after introduction into culture flasks.

[†] Each figure represents avg value obtained by pooling data from 3 separate experiments. Within each experiment at least 2 coverslips were read for each determination.

UV-treated phage regularly contained smaller numbers of brucellae per monocyte after 24 hours than cultures exposed to no phage or to heat-treated phage (Table II). (For any one of the experiments here summarized all of the phage-free and phage-exposed cultures originated from the same large sample of brucella-containing monocytes.) This effect of phage was also discernible by viable counts of brucellae harvested from phage-free and phage-exposed monocyte cultures, particularly when large numbers of brucellae were employed in the inoculum (Table III). The effect, though still discernible, was less pronounced in cultures with smaller inocula (Table IV).

The foregoing data are based on the fate of monocytes containing one or more brucellae. Also, in many, but not all, experiments with cultures exposed to untreated or UV-treated phage, an increased "0 class" was observed. That is, throughout the testing period the percentage of monocytes harboring one or more brucellae tended to be less (as low as 50%) in phage-exposed cultures than in phage-free cultures or in cultures exposed to heated phage (where the typical value was 80%). Since this difference was detectable as early as 2 hours after addition of phage, and since all the monocytes were equally infected initially, this difference between phage-exposed and phage-free cultures suggests that in the former an actual intracellular destruction of bacteria may occur.

TABLE III. Viable Counts of Intracellular *Brucella abortus,* SA-S, Harvested from Phage-Free or Phage-Exposed Monocyte Cultures with High Initial Brucella Counts.[*]

Time of exam.[†]	Treatment of cultures	Viable brucellae $(\times 10^5)/10^6$ monocytes[‡]
2	Phage-free	3.26 ± .32
	" -exposed	1.60 ± .15
24	" -free	12.66 ± 1.39
	" -exposed	1.56 ± .25
48	" -free	39.63 ± 5.61
	" -exposed	7.04 ± .82

[*] Cultures initiated with approximately 125 × 10^6 brucellae/10^6 monocytes.

[†] In hr after introduction into culture flasks.

[‡] Avg of duplicate determinations upon each of 2 cultures.

TABLE IV. Viable Counts of Intracellular *Brucella abortus,* SA-S, Harvested from Phage-Free or Phage-Exposed Monocyte Cultures with Low Initial Brucella Counts.[*]

Time of exam.[†]	Treatment of culture	Viable brucellae $(\times 10^4)/10^6$ monocytes[‡]
2	Phage-free	6.3 ± .37
	" -exposed	5.2 ± .34
	UV phage-exposed	5.4 ± .41
24	Phage-free	92.3 ± 8.62
	" -exposed	48.5 ± 6.33
	UV phage-exposed	51.1 ± 4.90

[*] Cultures initiated with approximately 25 × 10^6 brucellae/10^6 monocytes.

[†] In hr after introduction into culture flasks.

[‡] Avg of duplicate determinations upon each of 2 cultures.

Discussion. The finding that untreated, or UV-treated, brucellaphage is capable of interfering with intracellular and extracellular multiplication of *Br. abortus,* whereas heat-treated phage lacks this capability, suggests that the protein moiety of the phage is responsible. It remains uncertain in the case of the interference with the intracellular multiplication of brucellae: 1) whether the entire, untreated or UV-treated, phage particle is phagocytosed and subsequently enters into a direct bacterium-phage interaction, 2) whether only the active protein moiety diffuses to the susceptible bacterial site, or 3) whether the phage-protein, in conjunction with monocyte constituents, can cause changes in intracellular conditions that secondarily affect bacterial multiplication. The last hypothesis seems unlikely in view of the similarity between results obtained in extra- and intracellular systems. Also, preliminary tests, in which monocytes containing many brucellae were exposed to untreated phage, showed, 24 hours later, slight but consistent increases in phage titers (3.37×10^4 per 10^6 monocytes) over those obtained from brucella-free cultures (1.06×10^4 per 10^6 monocytes) or from cultures initially containing intracellular heat-killed brucella (1.67×10^4 per 10^6 monocytes). These observations would suggest that phage is actually phagocytosed and may interact with intracellular bacteria in the same manner as with extracellular bacteria. Finally, the observation that in phage-exposed cultures there is a decreased proportion of monocytes that contain stainable brucellae suggests the possible occurrence of additive effects between phage (or phage-protein) and cellular factors that can lead to the intracellular destruction of brucellae.

Summary. Studies on the interactions between brucellaphage and *Br. abortus,* either under extracellular conditions or when the bacteria resided intracellularly within guinea pig mononuclear phagocytes maintained *in vitro,* showed: 1) inhibitory effects of untreated or UV-treated phage on the multiplication of brucellae, and 2) a lack of such effects in the case of heat-treated phage. These findings suggest an association between brucellacidal, or possibly brucellastatic, effects and phage protein. It was demonstrated that the effects on intracellular bacteria can occur even when exposure to phage is delayed until 2 hours after phagocytosis of the bacteria.

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Creatine Synthesis After Creatinine Loading and After Nephrectomy.* (26137)

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Bloch and Schoenheimer(1) established that in mammals glycine and amidine (from arginine) react to form guanidinoacetic acid (GAA) and Du Vigneaud(2) demonstrated that the latter compound is methylated to form creatine. The two enzymes necessary for this synthesis are arginine-glycine transamidinase and guanidinoacetate methylferase. It had been thought for many years that the first of these enzymes was found only in the mammalian kidney(3). However, Walker (4) has demonstrated that the pancreas contains significant amounts of transamidinase, and could be a site of GAA synthesis. We have reported(5) that we were able to recover creatine-C^{14} after injecting nephrectomized rats with glycine-2-C^{14}, indicating that there is, in fact, a non-renal route of creatine synthesis. Horner(6) has reported essentially the same observation. However, in our study

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TABLE I. Effects of Nephrectomy and Creatinine Loading on Creatine Synthesis.

	Control			Experimental			% of control
	Avg cpm	SD	n	Avg cpm	SD	n	
Nephrectomy							
Glycine 2-C^{14}	1256		7	134		5	10.7
Glycocyamine 2-C^{14}	15898	4585	5	9944	1319	5	62.5
Creatinine loading							
Glycine 2-C^{14}	909	181	3	501	127	3	55.2
Glycocyamine 2-C^{14}	10333	727	3	7095	769	3	69.1

it was observed that rats made uremic by ureteral ligation showed a depression in amount of creatine synthesis, despite the presence of the kidneys. The present study was undertaken to determine whether uremia *per se*, interferes with creatine synthesis.

Methods. Three series of experiments were performed. In the first series Long-Evans rats weighing 300-400 g were lightly anesthetized with ether and bilaterally nephrectomized in a single stage procedure. Forty-eight hours later these animals were injected in the thigh with 25 μc of GAA-2-C^{14}. Seventy-two hours after nephrectomy the animals were sacrificed and 40 g of muscle were dissected from the carcass. Creatine was obtained from this tissue as potassium creatinine picrate by the technic of Foster, Schoenheimer and Rittenberg(7). The radioactivity of this material was then determined on a flow-counter with a micromil window using an infinitely thick sample of the potassium creatinine picrate dried to a constant weight. All counts were corrected for background and coincidence. Control animals were handled in an identical manner except that nephrectomy was not performed.

The second series of animals was loaded with creatinine by injecting 700 mg/kg of rat body weight intraperitoneally 2 times daily during an 8-hour period. All animals received a normal diet and water. After 3 days of such loading 50 μc of glycine-2-C^{14} were injected and the creatinine loading continued. Twenty-four hours later the animals were sacrificed and creatine-C^{14} determined as in previous experiment. Control animals were not injected with creatinine. The third series of animals was handled in an identical manner with the second, except that 10 μc of GAA-2-C^{14} were injected in place of the glycine-2-C^{14}.

Results are summarized in Table I. Nephrectomy reduced the utilization of glycine-2-C^{14} to 10.7% of control values, and was reported previously(5). Since this is the weighted average of 3 experiments, performed with modifications of dose of glycine-2-C^{14}, it is not possible to calculate a standard deviation. However, in the present series of experiments, nephrectomy reduced methylation of GAA by 37.5%, indicating that uremia not only decreases the renal component of GAA synthesis, but also impairs the non-renal methylation. The second and third phases of the study were performed to determine if the increased concentration of creatinine, inherent to renal failure, could result in reduced creatine synthesis and breakdown. It will be seen in the Table that incorporation of glycine was reduced 45% by creatinine loading, while methylation of GAA was reduced 31%.

Discussion. The original interest in this study was developed by the observation that with progressive, chronic renal failure the excretion of creatinine gradually decreased, and by extrapolation, was approximately 60% of normal when renal excretory function had decreased to zero(8). Three questions were posed: (1) Was this decrease due to an alternative pathway for creatinine excretion, (2) was it due to impaired renal synthesis of GAA, or (3) were other factors involved? Most of the alternative routes of creatinine excretion seemed to be excluded after specific examination(8). Sandburg, Hecht and Tyler(9) demonstrated that the product of the renal A-V difference in GAA concentration and the renal blood flow was inadequate to account for more than one-third of the daily production of creatine. This value compared favorably with the observed 40% decrease in creatinine excretion in total renal failure, and suggests that the decrease is actually due to loss of the renal component. However, in acute experiments(5,6) nephrectomy reduced the synthesis of creatine 78-94% when based upon C^{14} recovery from creatinine after glycine-C^{14} injection. Indirect evidence from our experiments, the demonstration that serum creatinine concentration falls after 5 days of dialysis, also suggested that extra-renal synthesis of creatine was being impaired. Therefore, the effect of high concentrations of creatinine on synthesis of creatine was investigated. The resultant experimental data indicate that increases in creatinine do, in fact, exert a profound effect upon creatine synthesis. Nephrectomy decreased GAA methylation by 37.5%, while creatinine loading reduced it by 30.9%. These values are remarkably comparable.

The summation of the loss of renal GAA synthesis plus the effect of uremic levels of creatinine should result in production of no more than one-fourth to one-third the normal amount of creatinine rather than the 60% which is observed in chronic renal failure. Since it is now established that there is an extra-renal route of creatine synthesis, and Walker has demonstrated the presence of both of the necessary enzymes in the pancreas(4,10), it is possible that under these circumstances there is an increase in pancreatic activity. It would be of interest to observe levels of transamidinase and methylferase in the pancreas and liver of animals made severely and chronically uremic as compared to normals. It is obvious that the inhibition of creatine synthesis observed in the acute nephrectomy experiments and following creatinine loading cannot operate in chronic uremia, since greater depression of creatinine excretion would result than that which is observed.

Recently Walker found that feeding 3% creatinine to rats reduced kidney transamidinase activity 12%, while feeding 3% creatine reduced transamidinase activity 66-68%(11). However, creatine feeding did not depress chick liver methyl-ferase activity, although creatine (but not creatinine) depressed chick liver transamidinase activity(12). Interestingly, one day of starvation or inadequate diet depressed the latter enzyme. Fitch, Hsu and Dinning(13) also report that creatine, but not creatinine, feeding reduces the activity of rat kidney transamidinase, with the maximum effect reached 2.5 to 4.5 days after institution of the test diet. Several possible explanations can be considered for the decreased creatine synthesis noted in our study. (1) Poor dietary intake may have depressed the methyl-ferase, as it does the transamidinase. Although originally matched in weight, creatinine-loaded animals averaged about 12 g less than controls at time of sacrifice. (2) The observed level of enzyme activity may not indicate the actual rate of reaction. (3) The rapid clearance of creatinine by intact kidneys may have prevented development of high levels of serum creatinine if the rate of intestinal absorption was slow. Intraperitoneal injection of 700 mg/kg body weight of creatinine caused serum creatinine to rise to approximately 40 mg%, with a rapid fall to normal levels (1 mg%) within 8 hours. However, total creatinine intake on a diet containing 2.5 to 3.0% (11,13) should be comparable to the amount in the daily intraperitoneal injections.

It seems probable that in chronic renal failure the rate of creatine synthesis is reduced by the amount of renal GAA component. In acute renal failure, and following loading with large amounts of creatinine, there is inhibition of both GAA formation and its subsequent methylation. This apparently occurs without depression of either of the essential enzymes. In chronic renal failure this inhibition seems to be overcome. It would be interesting to determine if there is an actual increase in either enzyme necessary for this response.

Summary. Previously, it was demonstrated that nephrectomy causes a 90% reduction in creatine synthesis in rats. In the present study, nephrectomy was found to cause a 37.5% decrease in methylation of guanidinoacetic acid (GAA)-2-C^{14}. Rats loaded with creatinine by intraperitoneal injection dem-

onstrated a 30% reduction in GAA methylation and a 45% reduction in total synthesis of creatine from glycine. It is concluded that chronic renal disease causes reduced creatine (and creatinine) synthesis both by loss of the renal mechanism and by interference with methylation. It is suggested that extra-renal (pancreatic?) synthesis may be relatively increased to partially compensate for these 2 inhibitory factors.

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Separation of Plasma Thromboplastin Antecedent (PTA) and Hageman Factor (HF) from Human Plasma.* (26138)

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Plasma acquires increased coagulability after contact with glass or certain other charged surfaces such as kaolin or celite. Following the discovery of patients deficient in plasma thromboplastin antecedent (PTA) (1) or Hageman Factor (HF)(2), these plasma clotting factors were shown to be involved in development of this increased coagulability. It has been postulated(3) that the glass surface "activates" HF enabling it to react with PTA and so initiate coagulation.

Waaler(4) found that clotting time of plasma lengthens markedly when it is treated with glass powder, the powder removed, and the plasma allowed to stand for several hours at 37°C. He called the product exhausted plasma (EP) and believed it was depleted of HF and PTA.

Others have attempted to purify HF and PTA, but these factors have not been separated. This communication describes the separation of HF and PTA by ion exchange chromatography. As shown below, each separated factor corrects the defect of its corresponding deficiency plasma. However, the combined purified factors fail to shorten clotting time of EP unless another as yet unidentified component of plasma is also present.

Methods. Our assays were based upon the kaolin clotting time technic of Margolis(5). The clotting system consisted of 0.1 ml of a suspension of cephalin-kaolin, 0.1 ml of a substrate plasma, 0.1 ml of the test substance, and 0.1 ml of 30 mM $CaCl_2$. The cephalin-kaolin suspension was prepared by combining equal parts of a 1/35 (v/v) suspension of cephalin in veronal buffer(6) and a 40 mg/ml kaolin suspension in physiological saline. The first 3 reagents were incubated together for 3 minutes at 37°C before the pre-warmed calcium was added and clotting time determined. The following substrate plasmas were used: PTA deficiency plasma for assay of PTA, HF deficiency plasma for the assay HF, or EP for assay of the com-

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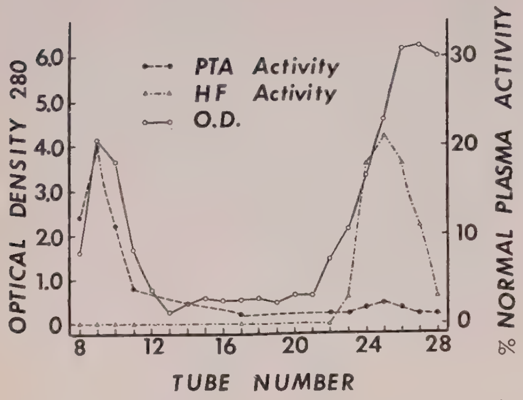

FIG. 1. PTA activity, HF activity, and protein concentration (OD at 280 mμ) of consecutive elution fractions from a DEAE cellulose column.

bined effects of HF and PTA.[†] The same systems were used with all contact surface removed (*i.e.*, without kaolin and with silicone-coated clotting tubes) to estimate prior glass activation. A dilution curve was obtained by substituting different concentrations of normal plasma into each assay system. The results when plotted on log-log paper produced a straight line graph from which relative concentration of unknown solutions could be estimated in % of normal plasma activity.

Separation of PTA and HF from intact[‡] normal plasma was carried out by using the ion exchange resin diethylaminoethyl cellulose (DEAE, California Corp. for Biochemical Research, Los Angeles). A constant pH of 7.12 was used; salt concentration was increased continuously from 0.05 M to 0.3 M by varying NaCl concentration in presence of 0.02 M sodium diethyl barbiturate buffer. The column was lucite with an ash free filter paper support. Neither lucite nor filter paper activated normal intact plasma. Fractions were collected in polyethylene tubes in a cold room.

Results. The results of a typical separation on DEAE are shown in Fig. 1. The maximum PTA activity coincided with the first protein peak as measured by ultraviolet absorption at 280 mμ, while the greatest HF activity immediately preceded the second protein maximum. All activities were corrected for the difference in volume between the plasma sample applied and the resulting column fractions. Fractions containing either PTA or HF were tested in specific assays for AHG, PTC, proconvertin, prothrombin-Stuart and proaccelerin. Less than 1% of normal plasma activity was found in each case.

TABLE I. Correction of EP by PTA and HF Deficiency Plasmas.

Test substance	Clotting time (sec.)
Citrate saline (in silicone)	195
PTA deficiency plasma	120
HF ” ”	63
Normal plasma	44

A study was made of the effect of PTA and HF fractions on clotting time of EP. Our EP, prepared by Waaler's method using celite(4), was shown by specific assays to contain adequate amounts of prothrombin, proconvertin, proaccelerin, Stuart factor, AHG and PTC. Its long clotting time was partly corrected by HF deficiency plasma but not by PTA deficiency plasma (Table I). Therefore, our EP was depleted primarily of PTA.

The prolonged clotting time of EP was

TABLE II. Failure of a Mixture of Purified PTA and HF to Correct EP.

Test substance*	Clotting time (sec.)
PTA deficiency plasma + HF deficiency plasma	61
PTA fraction + PTA deficiency plasma	68
PTA ” + citrate-saline	162
HF ” + HF deficiency plasma	68
HF ” + citrate-saline	181
PTA ” + HF fraction	158

* Quantities combined in equal parts in polyethylene tubes and 0.1 ml of the mixture used as test substance.

[†] PTA deficiency plasma was obtained from patients whose coagulation studies have been described(7). HF deficiency plasma was taken from a patient with classical clinical and laboratory findings. Dr. Oscar Ratnoff kindly substantiated the deficiency by cross correction experiments with this plasma in his laboratory. Mixing of HF and PTA deficiency plasma in our laboratory resulted in complete correction of their individual defects.

[‡] Intact plasma—plasma prepared in silicone-coated glassware or in plastic and therefore protected from contact with glass.

shortened either by a combination of PTA and HF deficiency plasmas or by each purified activity combined with its deficiency plasma (Table II). In contrast, addition of the combined purified activities did not shorten clotting time. These data suggest that plasma contains an additional activity which is missing from EP.

Summary. 1) Hageman factor and plasma thromboplastin antecedent have been separated from human plasma and each other by ion exchange chromatography. 2) The defect in "exhausted plasma" is not corrected by purified PTA, purified HF, or a combination of the two.

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Early Metabolic Effects of Human Growth Hormone.* (26139)

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The diabetogenic action of chronic administration of growth hormone is well established, both in experimental animals and in man. Insulin-like effects of the hormone have also been described, especially as a result of its acute administration(1) or of its presence *in vitro*(2,3). These effects have been related either to an insulin-like activity of the hormone itself or, *in vivo*, to a tropic effect of growth hormone upon the islets of Langerhans(4,5). In recent years, the physiologic importance of species differences in structures and function of growth hormone have become apparent(6,7,8), suggesting that previously observed effects should be reevaluated using growth hormone obtained from the same species. Although the metabolic effects of chronic administration of human growth hormone to human subjects with and without diabetes mellitus have been extensively described(9,10), the early metabolic effects have not been outlined as clearly.

In this report, an acute hypoglycemic effect of human growth in man will be described and related to changes in plasma free fatty acids described by Raben(11) and to measurements of serum insulin-like activity.

Materials and methods. Human growth hormone was prepared from pituitaries collected at autopsy according to the technic outlined by Raben(9). The potency of the preparation was tested against a sample obtained from Dr. Raben and exhibited approximately the same activity, mg per mg, using its effect upon plasma free fatty acids in man as activity index. When tested for contamination with ACTH, using the bioassay procedure of Nelson and Hume(12), 100 μg of the preparation contained less than 40 $\mu\mu$g purified ACTH.§

Growth hormone administration was carried out in 6 male human volunteers in apparent good health, ranging in age from 25 to 37 years. They were kept fasting overnight and at 8:00 a.m. a control blood sample was obtained. Human growth hormone dissolved in 3 ml of normal saline was then rapidly injected intravenously, subsequent blood samples being obtained 10, 20, 30, 60, 90 and 240 minutes after injection. No food intake was allowed during that time. The first subject received 4 mg of human growth hormone without side effects and 9 days later, 8 mg. Again, side effects were absent, but changes in the selected metabolic indices were greater. The 5 subsequent volunteers received therefore only a single dose of 8 mg of the hormone.

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† Trainee, U. S. Public Health Service.

‡ Research Fellow of Am. Diabetes Assn.

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Blood glucose was measured according to Somogyi's modification of the Nelson procedure(13) and plasma free fatty acids according to the method of Gordon(14). Insulin-like activity (ILA) was determined according to Martin, Renold and Dagenais(15) using oxidation of glucose-1-C^{14} to $C^{14}O_2$ by rat epididymal adipose tissue as index of ILA. In this procedure 6 segments of epididymal adipose tissue are obtained from each rat, of which 2 are needed for standards, thus leaving 4 segments for determination of unknowns. We have previously shown that the inter-assay error is greater than the intra-assay error(16). Accordingly, only 4 samples for determination of ILA were obtained in each subject at 0, 30, 60 and 240 minutes. All 4 samples were in each instance tested within the same assay on the same day. Assays were carried out on undiluted serum.

Before applying this assay for ILA to human serum after the intravenous injection of human growth hormone, it was necessary to establish whether the injected growth hormone preparation itself would interfere with the procedure. According to results reported by Read(17) with an immunoassay procedure for human growth hormone, normal plasma levels in man do not exceed 0.2 to 0.6 μg/ml. Furthermore, if the injected 8 mg of human growth hormone were entirely retained in plasma, the expected maximal level of injected growth hormone would be 3 μg/ml. Unpublished observations from this laboratory have established that concentrations of 0.5 and 5.0 μg/ml human growth hormone do not exhibit significant insulin-like or anti-insulin activity in the assay procedure used, either in buffer or in human serum. This is in contrast with the marked effects of larger concentrations of growth hormone which have been reported(18).

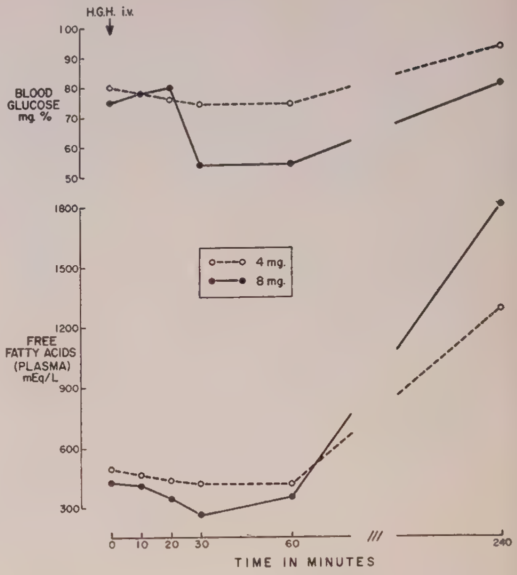

FIG. 1. Acute effects of human growth hormone given in a single inj. on blood glucose and free fatty acids (plasma) of a normal subject.

Results. The results obtained are shown in Fig. 1 and Table I. Fig. 1 demonstrates the relative effects of the smaller (4 mg) and larger (8 mg) dose of human growth hormone given at 9 day intervals to the same subject, while Table I summarizes all the results obtained with the higher (8 mg) dose. It is apparent from the data that intravenous administration of human growth hormone produced a very small initial rise in blood glucose, of doubtful significance ($P < 0.2$), then a marked and highly significant ($P < 0.001$) hypoglycemia with a mean blood glucose fall of 25 mg per 100 ml 30 minutes after administration of hormone. Free fatty acids first decreased ($P = 0.02$) then rose significantly ($P < 0.01$) to a mean level 3 times greater than control value 240 minutes after administration of the hormone. With continued fasting alone, for this relatively short period of 4 hours, plasma free fatty acids would not be expected to rise by more than 50%(11) and significant changes in blood glucose are unlikely. Furthermore, in

TABLE I. Acute Changes in Levels of Glucose, Free Fatty Acids and Insulin-Like Activity in Blood of 6 Normal Subjects after a Single Injection of Human Growth Hormone (8 mg i.v. at Time 0).

	Time in min.						
Subject	0	10	20	30	60	90	240
				Blood glucose (mg %)			
J.F.	75	78	80	54	54		80
J.C.	85	85	66	59	85		114
R.Z.	74	114	66	61	70		85
J.K.	73	70	58	47	43	62	77
A.F.	80	87	68	49	39	81	83
L.P.	85	94	80	54	44	78	85
Mean ± S.E.	79 ± 2	88 ± 7	70 ± 4	54 ± 2	56 ± 8	74	87 ± 3
				FFA-plasma (meq/l)			
J.F.	420	408	348	276	360		1800
J.C.	340	320	300	340	400		1240
R.Z.	635	830	528	540	595		1025
J.K.	360	340	260	200	340	500	800
A.F.	460	540	380	320	460	1100	1630
L.P.	600	320	320	340	360	780	2360
Mean ± S.E.	469 ± 55	460 ± 89	356 ± 42	336 ± 51	419 ± 43	793	1476 ± 255
				ILA-serum (μU/ml)			
J.F.	390			190	300		400*
J.C.	170			200	260		430
R.Z.	340			600	220		620
J.K.	170			120	170		220
A.F.	240			220	270		300
L.P.	140			130	120		210
Mean ± S.E.	242 ± 42			243 ± 73	223 ± 64		363 ± 63

* Indices of precision (lambda) of the individual assay runs follow: J.F., .18; J.C., .18; R.Z., .24; J.K., .20; A.F., .16; L.P., .22.

the subject who received 2 doses of growth hormone, both blood glucose and the plasma free fatty acid changes were considerably greater with the larger than with the smaller dose.

Values for ILA measurements are recorded together with the index of precision (lambda) for each assay day. Even with relatively satisfactory values for lambda, the 95% confidence limits for each single value range from approximately one-half the estimate to double the estimate. However, standard statistics may be correctly applied to each group of 6 values obtained at each of the times indicated in the 6 subjects, since no meaningless values were present as indicated by analysis of the assay characteristics of each day. ILA did not significantly vary from the control value in this group at either 30 or 60 minutes but showed a probably significant increase (P = 0.05) at 240 minutes. By way of comparison it may be pointed out that administration of glucose results in a 3- to 4-fold increase in serum ILA with the method used(16).

Three of the 6 subjects complained of a moderately severe headache between 3 and 5 hours after injection, without change in pulse rate or blood pressure. One of the subjects, furthermore, fainted when he suddenly assumed the vertical position at the end of experimental period. He reported that he had previously fainted with sudden changes in position. Ikkos, Luft and Gemzell(10) reported headaches and dizziness in 3 out of 4 patients given human growth hormone intramuscularly.

Discussion. These studies confirm the occurrence of early insulin-like effects of growth hormone preparations, even in the absence of species differences between preparations and test system and, more specifically, even for preparations of human growth hormone in *man*. The effects are insulin-like both in terms of hypoglycemia and in terms of an early decrease in circulating levels of free fatty acids. Failure to observe increased serum ILA at the time of maximum hypoglycemia and decrease in plasma free fatty acids suggests that the insulin-like effect of growth hormone preparations is neither secondary to a stimulatory effect on the pancreatic beta cells, nor associated with occurrence in serum of substances capable of potentiating the effect of serum insulin upon rat adipose tissue. It would seem more likely, therefore, that the early insulin-like action of growth hormone preparations is the result of intrinsic *in vivo* insulin-like activity of these preparations. Whether this insulin-like activity is directly related to growth hormone itself, or rather to presence of possible unrelated insulin-like components in the preparation is not clear. Huggins and Ottaway(19) and Bornstein and Hyde(20) have recently reported that peptides with insulin-like activity *in vitro* can be isolated from preparations of both ox and human growth hormone. Thus, presently available growth hormone preparations might be contaminated with an insulin-like peptide even as most crystalline insulin preparations are contaminated with glucagon. As in the case of glucagon, it will be necessary to establish whether the activity is an artefact of preparation or a substance of physiologic significance.|| It is, of course, impossible to rule out that an increase in serum ILA, too small to be detected yet sufficient to account for the hypoglycemia, did in fact occur.

The late (240 minutes) mild hyperglycemic effect of growth hormone, associated with the marked increase in free fatty acid levels, is consistent with the chronic "diabetogenic" action of human growth hormone in man (10). Increased ILA at that time could be considered as the secondary consequence of the rising level of blood glucose, although growth hormone induced stimulation of insulin-producing cells cannot be excluded. It may be of interest to consider the possibility that the early hypoglycemic effect of growth hormone contributes to some extent to the increased free fatty acid and blood glucose levels noted after 4 hours.

Summary. Human growth hormone, 8 mg, has been administered intravenously to 6 normal volunteers, and blood samples have been obtained before and from 10 to 240 minutes after injection of the hormone. The growth hormone preparation used exhibited a definite early insulin-like effect both in terms of blood glucose and plasma free fatty acid levels. This was not associated with a change in serum insulin-like activity, as measured with rat epididymal adipose tissue. Four hours after growth hormone had been given, there was a mild hyperglycemic effect, and a marked increase in plasma free fatty acids, together with a small but significant elevation in insulin-like activity of serum. These observations are interpreted as compatible with an intrinsic *in vivo* insulin-like activity of the growth hormone preparation used, which could be either a property of the growth hormone molecule itself, or represent the activity of unrelated substances present in the preparation.

|| During preparation of this manuscript the authors have become aware of the studies of Domingues, Greenberg, Pazianos, Ray and Pearson, *Acta Endocrinol.* (Copenhagen), 1960, Suppl. 51, p. 241. These authors observed an early decrease in blood glucose and plasma free fatty acid levels following intravenous administration of 5 mg human growth hormone to *both diabetic and non-diabetic subjects.* They concluded that "the hypoglycemic effect of growth hormone does not appear to be due to insulin release, since it occurs in the juvenile-type diabetic patient." This observation further substantiates the interpretation given to our results reported here. An early decrease in plasma free fatty acids has also been described by Beck and his collaborators, *Ciba Found. Colloquia on Endocrinology*, 1960, v13, 164.

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Passive Transfer of Delayed Sensitivity to the Newborn Rabbit.[*] (26140)

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Recent studies of the immune capacity of the human newborn infant have demonstrated that the newborn infant is unable to accept or to manifest a passive transfer of delayed allergy(1). A similar unresponsiveness to passively transferred delayed allergy in the newborn guinea pig was demonstrated by Waksman and Matoltsy(2). They were unable to transfer delayed sensitivity to tuberculin to newborn guinea pigs with peritoneal exudate leukocytes, but were uniformly successful with such transfers to mature guinea pigs. Parallel limitations in capacity of the newborn human and newborn rabbit have been demonstrated by several workers(3,4,5,6). Data presented here show that like newborn infants newborn rabbits are limited in this capacity to express or to accept passively transferred delayed allergy.

Methods and materials. The animals used in the experiment were New Zealand white rabbits, newborn and mature. The newborn rabbits were 7, 14, 21, and 28 days of age, and the mature rabbits weighed 5 to 6 lb.

Delayed sensitivity was induced by infecting adult animals with Demateo strain of streptococcus. The Demateo streptococcus is group A, type 12 strain of beta-hemolytic streptococcus which was originally obtained from a patient with acute glomerulonephritis. This strain produces large quantities of streptokinase and streptodornase B. One-tenth cc of an 18-hour broth culture of the Demateo strain streptococcus was injected into each hind foot pad of the 5-6 lb rabbits.

These rabbits were skin tested 7 to 10 days after infection to determine their degree of sensitivity. Skin testing was done with 0.01 cc of physiologic (8.5%) saline containing 100 units of streptokinase and 3.3 units of streptodornase[‖] injected intradermally in the inner surface of the rabbit's ear. As a control, 0.01 cc of physiologic saline was injected in the opposite ear. The skin testing sites in the ears were examined 18-24 hours later for induration and erythema. The skin reactions were read on the basis of square millimeters of erythema and induration, as follows: 0-9 mm^2 – no transfer, 10-19 mm^2 – 1+, 20-39 mm^2 – 2+, 40-59 mm^2 – 3+, 60-79 mm^2 – 4+, over 80 mm^2 – 5+. The same rating system was used for grading the passive transfers.

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TABLE I. Results of Passive Transfer of Delayed Sensitivity to Newborn and Control Rabbits.

Exp.	Age of newborn (wk)	Intensity of transfer to Newborn	Control
1	1	0	5+
2	1	0	2+
3	1	0	4+
4	1	0	0
5	1	0	1+
6	1	0	3+
7	1	0	3+
8	2	0	0
9	2	0	1+
10	2	0	1+
11	2	1+	2+
12	2	0	5+
13	2	0	2+
14	2	0	0
15	2	0	0
16	2	0	3+
17	2	0	2+
18	2	0	0
19	3	2+	0
20	3	0	2+
21	3	4+	1+
22	3	0	5+
23	3	0	0
24	3	0	1+
25	3	0	5+
26	3	2+	2+
27	3	3+	5+
28	4	5+	5+
29	4	5+	5+
30	4	5+	5+

Preparation of leukocytes for transfer. The rabbits giving a positive skin test were sacrificed. The spleen and the 2 popliteal nodes were removed. Leukocytes were separated from these organs by teasing with dissecting needles or by forcing the whole organ through a 40 mesh stainless steel sieve which had been coated with Arquad® to prevent agglutination of leukocytes. The resultant material was suspended in 40 cc of saline and permitted to stand for 10 minutes to allow settling of the coarse particles of tissue. The supernatant fluid was withdrawn and centrifuged for 12 minutes at 1500 rpm. The cell button containing a mixture of red and white cells was resuspended in a standard volume, usually 3 or 4 cc of saline. Aliquots of the leukocytes were injected by the same routes—intravenous, intraperitoneal, or subcutaneous—into a newborn and a mature control rabbit in each experiment.

Skin testing. After transfer of white blood cells, the animals were skin tested with 0.01 cc of physiologic saline containing 1000 units of streptokinase and 33 units of streptodornase. When the cellular transfer was done intravenously, the skin testing was done immediately after injection. When the transfer was done intraperitoneally or subcutaneously, the skin testing was carried out 24 hours after the transfer of cells.

Results. In Table I are the results of several experiments with rabbits aged 1-4 weeks. At the youngest age, no newborns accepted passive transfer of delayed sensitivity, but with increasing maturity, larger proportions were able to demonstrate this phenomenon.

Newborn rabbits aged 7 days are uniformly unable to accept passive transfer of delayed sensitivity. At the age of 14 days, one of 11 accepted the passive transfer and with increasing maturation, the acceptance rate rose to 4 of 9 at 21 days and 3 of 3 at 28 days (Fig. 1). Passive transfers of delayed sensitivity to mature control animals were successful in 24 of 30 attempts.

Discussion. These data indicate that the newborn rabbit is similar to the newborn human infant in its inability to accept the passive transfer of delayed sensitivity when compared with the immunologically mature rabbit. The data do not indicate the basis for

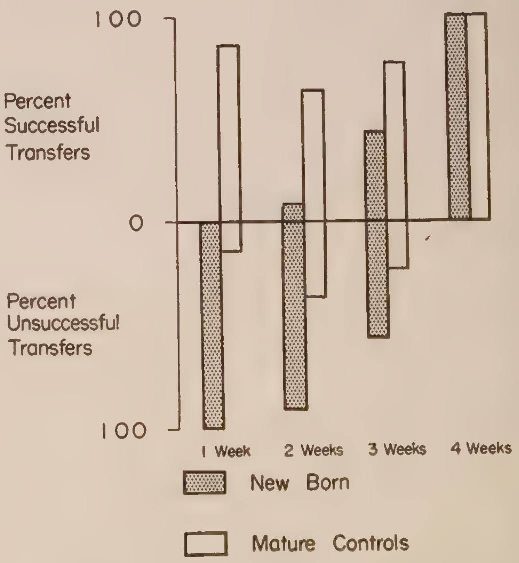

FIG. 1. Progressive maturation of newborn rabbit's capacity to sustain the passive transfer of delayed hypersensitivity.

the failure of the response. This could be the result of the newborn rabbit's inability to sustain the transferred cells, to coordinate the cellular and vascular elements responsible for expression of delayed hypersensitivity, or to perform an essential step in development of the hypersensitive state. The capacity to accept passively transferred delayed sensitivity appears to mature by 4 weeks of age, with gradual transition between 2 and 3 weeks of age. This phenomenon may be common to all animals expressing delayed sensitivity. It should be studied further, not only because of its possible significance in understanding the basis for delayed allergy, but because of important implications which this deficiency may have in understanding the relative inability of the newborn to defend himself against infection and toxic stimuli.

Summary. The newborn rabbit is limited in its capacity to accept the passive transfer of delayed hypersensitivity, as is the case in the human newborn. The ability to accept passively transferred delayed sensitivity develops by 4 weeks of age in newborn rabbits.

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Biosynthesis of Squalene by Rat Preputial Gland.* (26141)

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During a study of effects of hormones on the rat preputial gland, it was found that this gland appears to synthesize and accumulate squalene. Preputial glands are present in both sexes of several species of rodents as a pair of small, clubshaped organs lying along the urethra, and emptying their oily secretion into the preputial sac. The function of these glands is unknown, but they contain large amounts of β-glucuronidase and acid phosphatase. A mouse preputial gland tumor incorporated C^{14} from acetate into sterols when incubated *in vitro* while normal mouse preputial glands were less active in this regard(1). Administration of androgens to rats of either sex causes a marked increase in size of preputial glands, whereas, hypophysectomy leads to atrophy. The preputial gland is similar in microscopic structure to the cutaneous sebaceous glands, and in the rat, its responses to administration of hormones are thought to be similar to those of the sebaceous glands(2).

Methods. Preputial glands were obtained by excision immediately after death (by blow on head) from normal Sprague-Dawley male rats which were 6 to 7 weeks old and weighed 160-200 g. The glands, which weighed from 20 to 75 mg, were dissected free of fat and other tissue, and were incubated whole or split longitudinally in 1 ml of Krebs-Ringer phosphate buffer (pH 7.4) to which had been added sodium acetate 1-C^{14} (12.5 μc of C^{14} in 25 μmoles of sodium acetate). Incubation was carried out in a Dubnoff shaking incubator at 37°C under a flow of oxygen and shaking at 100 strokes per minute for 3 hours. The tissue and incubating fluid were then heated in a steam bath overnight with alcoholic potassium hydroxide solution in tubes sealed under nitrogen. The nonsaponifiable lipids were extracted with petroleum ether and separated into squalene and sterol fractions by column chromatography on alu-

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TABLE I. Incorporation of C^{14} from Acetate into Squalene and Sterols by Various Tissues *In Vitro.*

Tissue	No. of samples	cpm/mg tissue	
		Squalene	Sterol digitonide
Rat preputial glands	25	78 (52 -108)*	2.8 (1.4- 6.2)
Human skin	34	32 (10 - 64)	1.4 (.4- 6.6)
Mouse preputial glands	10	1.2 (.5- 2.5)	5.3 (3.4- 6.6)
Rat liver	10	1.4 (.6- 2.5)	22 (12 -39)

* Range of values.

mina. The squalene was counted as an infinitely thin layer in cup planchets, and the sterols as an infinitely thin layer of the digitonide on filter paper in a thin end window gas flow counter. Results were expressed as counts per minute, per mg, of original tissue. It is recognized that this method of expressing the radioactivity does not give absolute comparisons of amount of incorporation into each lipid fraction, but it is felt that it is adequate to show differences in incorporation into these 2 lipids by different tissues. Further details of the method have been described(3) in which human skin was studied. Since incorporation of C^{14} into squalene was of special interest, the "squalene fraction" was further examined. In previous studies, it was found that the squalene fraction obtained by alumina chromatography of the nonsaponifiable lipids, contained squalene in good yield without detectable radioactive contaminants. To identify this fraction more accurately, the hexahydrochloride derivative was made. Commercial shark squalene was added to the radioactive squalene fraction and after rechromatography on alumina, the hexahydrochloride of the mixture was prepared(4). After careful washing and recrystallization, specific activity of the squalene hexahydrochloride was found to be 90% of that of the original squalene mixture, indicating that the radioactive squalene fraction was truly squalene. A larger amount of untagged squalene (about 4 mg) was prepared from 2.6 g of rat preputial glands (25 rats) in the same manner as the individual gland studies. The infra red spectrum of this substance (studied as a film without solvents)[†] was identical with that of shark squalene, and also with the spectrum for redistilled shark squalene(5).

Results. Preputial glands from young male rats incorporated C^{14} from acetate into squalene more actively than any other tissue that we have studied. Conversely, there was relatively little incorporation of C^{14} into sterols (Table I). (Preputial glands from female and male rats of all ages showed the same pattern, though actual amount of incorporation into squalene varied from 25 to 110 cpm/mg). Human skin exhibits the same pattern of incorporation of C^{14} into squalene and sterols. Representative samples of breast and scapular skin obtained at operation or biopsy are included in the table. Previous differential studies demonstrated that incorporation of C^{14} into squalene occurred mainly in the dermis, the site of sebaceous glands, while the epidermis carried on the more usual pattern of incorporation of C^{14} predominately into sterols(3). Mouse preputial glands (from young males, 26-30 g) on the other hand, followed the pattern of most other mammalian tissues in that they incorporated C^{14} more actively into sterols than squalene (Table I). Rat liver, which was incubated as 100 mg cubes, is included for comparison and shows the well known pattern of incorporation mainly into sterols. It is generally felt that squalene is a normal step in the synthesis of sterols by most tissues, and thus little of this substance accumulates. It is not clear why squalene accumulates in preputial glands or in human skin. It is possible that there is absence of the factors necessary for conversion of squalene to sterols or that inhibitors of the process are present in human skin and preputial glands. It is also possible that the squalene produced by these tissues is biologically inactive because of some small difference in chemical structure. A final possibility is that the initial content of squalene in these tissues acts as a trap for radioactive squalene formed during the course of sterol synthesis. If this is so, the original source of their squalene is unexplained.

[†] Infra red spectrography was kindly performed by Dr. S. Wang, Dept. of Physiology, Tufts Univ. School of Med.

Summary. Preputial glands from rats, when incubated with C^{14} acetate show a marked incorporation of C^{14} into squalene, a sterol precursor, and little into sterols. In this regard, the preputial glands differ from many other mammalian tissues which show incorporation of C^{14} mainly into sterols and little into squalene. Human dermis, which contains sebaceous glands, is another mammalian tissue which shows preferential accumulation of the C^{14} into squalene. The mechanism of the difference is unknown, though it seems likely that factors necessary for conversion of squalene to sterols are missing in these tissues, or that an inhibitor of this reaction is present.

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Studies of Bovine Erythrocytes in Anaplasmosis. I. Flocculating Properties of Stromata in Water.* (26142)

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The erythrocytic membrane has been shown to be intimately connected with phospholipids as structural components(1). Chemical analyses of stromata in various anemias have shown that there are alterations in content of phospholipids(2,3). New evidence of surface structural changes obtained by visual methods and involving the phospholipids of the erythrocyte in bovine anaplasmosis was collected while studying the mechanism of the anemia produced. Observations showed that the flocculating characteristics of erythrocytic stromata prepared from blood of uninfected calves and calves infected with *Anaplasma marginale* were different from each other when suspended in water.

Materials and methods. Stromata were prepared from erythrocytes of 14 normal intact, 6 splenectomized uninfected, and 18 splenectomized infected calves. The latter group of animals was inoculated by the subcutaneous route with 10 ml of infective blood having approximately 30% of the erythrocytes showing anaplasma bodies. Jugular vein blood samples were collected periodically in 50-ml amounts in 0.3 ml heparin sodium solution (1,000 U.S.P. units/ml). The erythrocytes were thoroughly washed and packed in 0.85% sodium chloride solution by centrifugation at 700 × g for 30-minute intervals at 4°C. They were then mixed and lysed in 20 volumes of distilled water at 4°C which was saturated with carbon dioxide. The carbon dioxide saturated distilled water possessed a pH of 3.8 at this temperature. The material was allowed to settle in tall cylinders overnight at 4°C and the clear red supernatant fluid was aspirated off. The fluffy appearing stromata were poured into 250-ml centrifuge bottles and washed in unbuffered distilled water by centrifugation at 700 × g for 10-minute intervals at 4°C. This unbuffered distilled water had a pH of 5.4. Stromata have been prepared by lysing cells and

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flocculating and washing the material in a sodium citrate buffer at pH 5.5(4). Washing in cold unbuffered distilled water at pH 5.4 was continued until the supernatant fluid appeared devoid of hemoglobin. After the last washing approximately 10 ml of unbuffered distilled water at pH 5.4 were then added to initially suspend the sediment by shaking and the centrifuge bottle was completely filled with distilled water and allowed to stand at room temperature. Photographs were taken 10 minutes after the stromata were suspended.

In this laboratory the use of buffered and unbuffered salt solutions produced decreased yields of bovine erythrocytic stromata. This was due to increased solubility of stromata, *per se*(5) and also specifically to increased solubility of lipoidal materials from the stromata(6,7). The supernatant fluids in preparations made with the aid of salt solutions remained cloudy in all instances. The precipitation method of preparing bovine stromata using cold unbuffered carbon dioxide saturated distilled water at pH 3.8 and subsequent washing with cold unbuffered distilled water at pH 5.4 produced adequate yields of material by which the flocculation characteristics could be observed.

Subsequent extraction of total phospholipids from desiccated stromata were made with 3:1 ethanol:ether mixtures(8). The phospholipids included cephalin, lecithin, and sphyngomyelin(3). Estimations of total phospholipids were made on these extracts by using the chemical method of Fiske and Subbarow for phosphorus(9). The factor, total phospholipid phosphorus × 25, was used to convert the values to total phospholipids(10).

Results. Representative examples of the altered flocculating properties are shown (Fig. 1). In preparations of stromata from normal cells the material agglutinated rapidly and settled in a period of 5 minutes (Fig. 1, a). Stromata prepared from erythrocytes showing anaplasma bodies exhibited varying amounts of suspended and finely divided material which remained in suspension for relatively long periods of time (3-18 hr, Fig. 1, b-f).

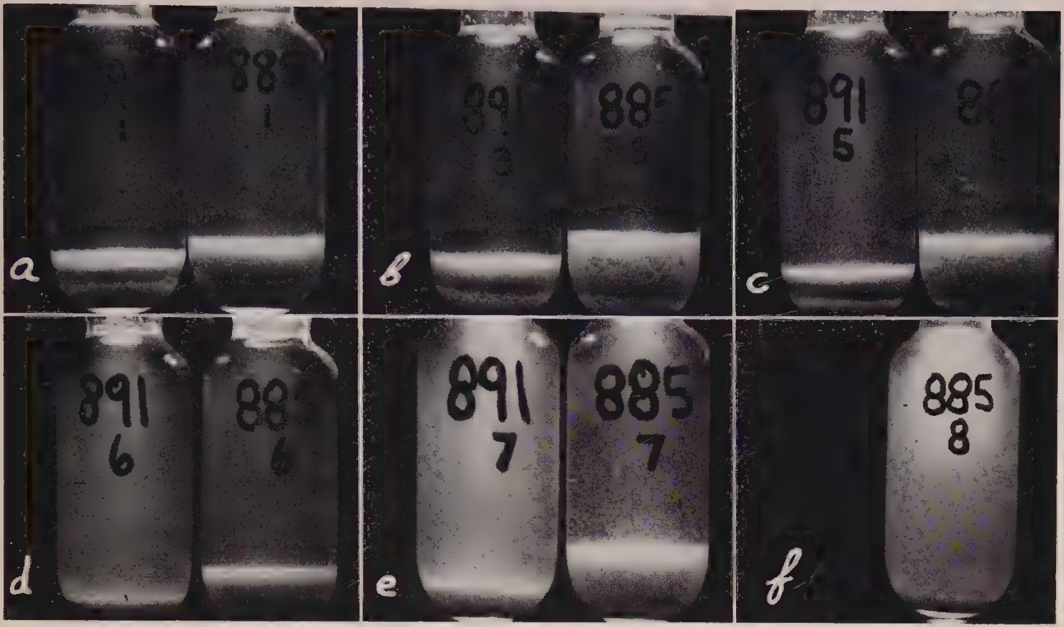
The modified flocculating properties began to manifest themselves shortly after the anaplasma body count had reached approximately one-half of its peak value or when the erythrocytic count was just beginning to decrease (Fig. 1, c). An increase in the osmotic fragility index was also observed at this time.

Concentrations of total phospholipids of stromata exhibiting large amounts of suspended matter were as low as 60% of preinfection values (14% down to 6%). It has been reported that normal bovine erythrocytic stromata contain 16% total phospholipids, whereas embryonic bovine stromata contain 11.9% total phospholipids(11). Erythrocytes showed high osmotic fragility indices and in many instances lysed spontaneously in isotonic 0.85% sodium chloride solution. Erythrocytic counts were extremely low (1.5-3.0 million/mm^3) during this time. Anaplasma body counts had already reached peak values and were decreasing and approaching a low level of less than 2%.

Recovery patterns in the flocculating characteristics of stromata were also observed. When erythropoiesis and recovery from the disease were rapid as evidenced by an increasing number of erythrocytes and a low or negative anaplasma body count, the stromata showed progressive increases in ability to aggregate and settle rapidly. This return to normal flocculating properties was also accompanied by an increase in level of total phospholipids of the stromata.

In studying animals undergoing prolonged periods of convalescence fluctuations in anaplasma body counts occurred. Erythrocytic counts showed some variations also, but a trend in an increase in numbers was observed. Although suspensions of stromata appeared cloudy during this phase of the disease in these animals the fragility index was normal. In each of these instances the animals died. Total phospholipid content of the stromata was below normal in all cases in which this phenomenon occurred.

Discussion. A possible explanation for this phenomenon seems to be that a change in isoelectric point of the stromata has taken place which in turn has affected their hydration affinity. The change in chemical com-



	Designation on centrifuge bottle					
Fig. 1 letter	Animal No.	Sample No.	ABC	ER	FI	Days after inoculation
a	891	1	negative	9000	.67	5
b	891	3	1.0	7300	.70	9
c	891	5	36.0	6050	.73	14
d	891	6	49.0	3350	.76	16
e	891	7	60.0	2425	.76	17 (died)
a	885	1	negative	7150	.67	5
b	885	3	1.0	6425	.70	9
c	885	5	25.0	6125	.73	14
d	885	6	42.0	4850	.76	16
e	885	7	50.0	3600	.76	17
f	885	8	69.0	1600	.85	19 (died)

FIG. 1. Anaplasma body count in % erythrocytes showing bodies (ABC), erythrocytic count/mm^3 in thousands (ER), fragility index in highest % sodium chloride solution showing trace of hemolysis (FI).

position, in this case decreased content of acidic lipids, namely the phospholipids, has effected a change in isoelectric point toward a higher pH value. The isoelectric point of stromata with lipids experimentally extracted has been shown to be at least 3 pH units higher than intact erythrocytes(12). It has also been shown that cells having large amounts of lipoidal material in the surface have low isoelectric points and are capable of becoming the least hydrated while their ability to become aggregated is great(13). Normally the isoelectric point of bovine erythrocytic stromata is approximately pH 3.4(13). The stromata preparations would then tend to remain in suspension for relatively long periods under the conditions cited herein.

The altered flocculating properties, specifically the tendency of stromata showing a decreased total phospholipid content to remain in suspension, might be assumed to be due to presence of reticulum from reticulocytes and immature cells. Ponder(14) has reported that reticulocytes contain more lipid than normal erythrocytes but that total lipid and phospholipid does not differ appreciably from normal when calculated on the basis of the red cell surface which is increased in the reticulocyte. Density of the immature cell is also less so it presumably contains more water, although it is stated to be stickier than the orthochromatic cell(14). The possibility that reticulocytes and other immature cells may be responsible for the different flocculating characteristics can be discounted by the fact that these immature cells appear shortly after the peak in anaplasma body count has been reached(15). In this case the percentage rise is from a normal of approximately 0.5% to approximately 5% after the peak. Our unpublished observations have indicated that a small number of immature cells also appear at approximately the same time. The change in flocculating properties described herein first appeared prior to the peak in anaplasma body count and continued thereafter only at a time when the content of total phospholipids of the erythrocytic stromata was lower than normal.

As further evidence against immature cells being responsible for this phenomenon it has been found that reticulocytes and immature erythrocytes are less fragile than mature cells (16). In the studies reported here fragility indices were usually high during the appearance of immature cells after the anaplasma body count decreased to a low level, indicating that the cell population as a whole was more susceptible to osmotic lysis.

The differences observed in stromata of animals undergoing long periods of convalescence indicated that imperfect or malformed cells were being produced. Possibly there was a partial disorientation of cell membrane ultrastructure. Hansard and Foote(17) have found that there are malformed cells produced in anaplasmosis during a study of the anemia using Fe^{59}. Although these new cells which possessed a lowered content of total phospholipid had an abnormal structure, they were still physically sound as to osmotic fragility. These imperfect cells may be responsible for an impaired erythrocytic function which indirectly causes anoxia and death even though anaplasma body counts are decreasing and erythrocytic counts are increasing.

It would appear reasonable that this phenomenon may manifest itself in other anemias especially those in which phospholipids are affected. It may have application, also in determination of the type of anemia when chemical composition of the stromata is changed.

These studies are being continued with particular emphasis on partition of phospholipids and other chemical and physical properties of diseased erythrocytes.

Summary. Differences in flocculating characteristics of erythrocytic stromata prepared from blood of uninfected calves and calves infected with *Anaplasma marginale* are described. The differences in flocculating properties are due to a change in isoelectric point brought about by the altered total phospholipid content.

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Failure of Oxytocin to Initiate Mammary Secretion in Rabbits or Rats.* (26143)

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Benson and Folley(1) demonstrated that oxytocin inhibited mammary involution in postpartum rats after litter removal, and this has been confirmed(2). Since it was previously shown that prolactin could similarly inhibit mammary involution(3), the British workers hypothesized that the effects of oxytocin were mediated through anterior pituitary release of prolactin. We have questioned this hypothesis for several reasons, including the observation that oxytocin did not initiate lactation in animals with developed mammary glands(4-5). Recently however, Haun(6) reported successful induction of lactation in mature New Zealand White female rabbits with multiple injections of oxytocin for 2 days, after initially priming them with estrogen for 21 days. We have attempted to duplicate his procedure, and in addition have given oxytocin to pseudo-pregnant rabbits and estrogen-primed rats. Oxytocin failed to initiate mammary secretion in these animals.

Methods. Five groups of mature virgin New Zealand White female rabbits, weighing 3.5-4.5 kg, were injected subcutaneously with 0.1 mg estradiol in corn oil on alternate days for 20 days. On the 21st and 22nd days, a control group was injected intraperitoneally with physiological saline 4 times daily; and 3 groups, with .3, .6 and 1.2 IU oxytocin‡ respectively, given intraperitoneally 4 times daily. Mammary glands were inspected for lactation *via* mid-ventral incision on the 25th day. In another experiment, pseudopregnancy was induced in mature virgin rabbits of the same strain and weight by a single intravenous injection of 100 IU chorionic gonadotropin. This results in excellent mammary lobule-alveolar development by the 12th day. On days 12-16 of pseudopregnancy, one group received no further treatment and served as controls; another group was injected subcutaneously with 10 mg prolactin§ once daily; and 3 groups received .3, .6 and 1.2 IU oxytocin respectively, given intraperitoneally 4 times daily. On the 17th day mammary glands were examined for lactation *via* mid-ventral incision.

For the rat experiment, mature virgin females of the CFN Carworth strain, weighing 180-220 g, were injected subcutaneously with 10 μg estradiol in corn oil once daily for 10 days to develop a lobule-alveolar system. For the next 5 days, separate groups were injected with saline, oxytocin, vasopressin, prolactin, ACTH,‖ and prolactin and ACTH, respectively. On the 16th day, the rats were killed and the right inguinal mammary gland was removed, fixed and stained by standard histological technics(2), and examined for secretion microscopically.

Results. In the estrogen-primed rabbits, 2 of 6 control rabbits (Group 1) had a slight amount of milk in the ducts and there was little or no lobule-alveolar development. Only 2 of 18 oxytocin-injected rabbits (Groups 2-4) had a small amount of milk in the ducts, and the mammary glands were no better developed than in controls. In the pseudopregnant rabbits, 2 of 6 controls (Group 5) showed a slight amount of serous secretion in the ducts, whereas mammary glands of all 6 prolactin-injected animals (Group 7) were distended with milk which could easily be expressed from the nipples. Oxytocin (Groups 6-9) did not initiate milk secretion at any

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‡ Oxytocin was supplied through courtesy of the late Dr. D. A. McGinty, Parke, Davis and Co., Detroit.

§ Prolactin (1 mg = 15 IU) was donated by Endocrinology Study Section, N.I.H.

‖ ACTH gel (40 USP units/cc) was supplied through courtesy of Dr. R. O. Stafford, Upjohn Co., Kalamazoo, Mich.

TABLE I. Effects of Oxytocin[*] and Prolactin[†] on Initiation of Lactation in Estradiol-Primed[‡] and Pseudopregnant[§] Rabbits.

Group and No. of rabbits	Treatment	No. with milk	Remarks
	Estradiol-primed rabbits (examined on 25th day)		
1 (6)	Controls, saline 4× daily on days 21-22	2	Slight duct secretion
2 (6)	.3 IU oxytocin *idem*	1	*Idem*
3 (6)	.6 IU oxytocin "	1	"
4 (6)	1.2 IU oxytocin "	0	No secretion
	Pseudopregnant rabbits (examined on 17th day)		
5 (6)	Controls, 17th day pseudopregnancy	0	Serous secretion in 2
6 (6)	10 mg prolactin daily on days 12-16	6	Copious milk throughout
7 (3)	.3 IU oxytocin 4× daily on days 12-16	0	Serous secretion in 1
8 (3)	.6 IU oxytocin *idem*	0	*Idem*
9 (3)	1.2 IU oxytocin "	0	No secretion

[*] Oxytocin (Parke-Davis) was inj. intraper.
[†] Prolactin (NIH, 15 IU/mg) was inj. subcut.
[‡] .1 mg estradiol inj. subcut. on alternate days for 20 days.
[§] Pseudopreg. induced by single intrav. inj. of HGG.

dose level, although 2 animals showed slight serous secretion in the ducts (Table I).

In the rats (Table II), mammary glands of controls (Group 1) regressed to a bare duct system, as did mammary glands of oxytocin (Groups 2-5) and vasopressin (Groups 6-7) rats. No secretory activity was observed in these rats. Neither prolactin (Groups 8-9) nor ACTH (Groups 10-11) alone induced secretion, although mammary lobule-alveolar structure was well maintained by prolactin. When both prolactin and ACTH were administered (Group 12), mammary maintenance and secretion were observed in 4 of 5 rats. This shows that both prolactin and ACTH are necessary to initiate mammary secretion in estrogen-primed rats, as reported previously(7).

TABLE II. Effects of Oxytocin,[*] Vasopressin,[†] Prolactin,[‡] and ACTH[§] on Initiation of Lactation in Estradiol-Primed Rats.

Group and No. of rats	Treatment for 5 days after estradiol	No. with secretion
1 (10)	Controls, saline 2× daily	0
2 (5)	1 IU oxytocin 2× daily	0
3 (5)	1 " " 4× "	0
4 (5)	2 " " 2× "	0
5 (5)	10 " " " "	0
6 (5)	1 " vasopressin 2× daily	0
7 (5)	5 " " " "	0
8 (5)	1 mg prolactin 2× daily	0[‖]
9 (5)	2 " " " "	0[¶]
10 (5)	1 IU ACTH " "	0
11 (5)	2 " " " "	0
12 (5)	1 mg prolactin + 1 IU ACTH 2× daily	4

[*] [†] Oxytocin and vasopressin (Parke-Davis) were inj. subcut.
[‡] Prolactin (NIH, 15 IU/mg) was inj. subcut.
[§] Depot-ACTH gel (The Upjohn Co.) was inj. subcut.
[‖] [¶] Mammary lobulo-alveolar structure was maintained.

Discussion. Oxytocin failed to initiate mammary secretion in estrogen-primed or pseudopregnant rabbits in contrast to the report by Haun(6), although his procedure in respect to strain and size of rabbit, hormone dosage and length of treatment was duplicated as closely as possible. A private communication from Folley[¶] also states that "a recent attempt to repeat Haun's results on New Zealand White rabbits by Benson has failed completely." It is possible that the limited mammary secretion noted by Haun was due to estrogen rather than oxytocin stimulation. Neither oxytocin nor vasopressin inhibited mammary involution or initiated secretion in estrogen-primed rats. By contrast, injections of prolactin alone inhibited mammary involution, and injections of both prolactin and ACTH initiated mammary secretion in estrogen-primed rats. Thus oxytocin did not induce prolactin release in either rabbits or rats.

Other work also indicates that prolactin release from the pituitary is independent of oxytocin secretion. Thus (a) hypothalamic lesions in the rat inhibit oxytocin release and prevent litters from obtaining milk, although milk production and hence prolactin secretion continue in a normal fashion(8); (b) single pituitaries transplanted to the kidney of rats secrete greater than normal amounts of prolactin and initiate mammary secretion, despite absence of obvious oxytocin stimulation(9); (c) whereas suckling evokes an immediate decrease in pituitary prolactin content, indicating discharge of this hormone, injections of oxytocin are ineffective in this respect(4). Benson and Folley's(1) demonstration that oxytocin inhibits mammary involution in postpartum rats can be explained by its direct action on the mammary gland(5,10).

[¶] Letter dated March 30, 1960.

Summary. Oxytocin failed to initiate lactation when injected 4 times daily into 45 mature female New Zealand White rabbits initially primed with estradiol or made pseudopregnant to develop their mammary glands. Prolactin injections initiated copious milk production in pseudopregnant rabbits. 2. Neither oxytocin nor vasopressin inhibited mammary involution or induced secretion in estrogen-primed rats, whereas injections of prolactin alone retarded mammary involution and addition of ACTH initiated mammary secretion. 3. It is concluded that oxytocin does not induce prolactin release in rabbits or rats.

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Received July 18, 1960. P.S.E.B.M., 1960, v105.

Effect of Diazoalkane-yielding Compounds on Strains of *Escherichia coli* Resistant to 1-Methyl-3-nitro-1-nitrosoguanidine.* (26144)

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1-Methyl-3-nitro-1-nitrosoguanidine (NG) has anticancer activity in mice(1,2,3) and antibacterial activity *in vitro*(4,5). Two mutants of *Escherichia coli* strain S which are resistant to NG have been selected. The first-step resistant mutant S/Ng 1 was selected from the wild type, strain S. The second-step mutant S/Ng 2 was selected from S/Ng 1(6). It was believed that some insight into the mechanism of action of NG could be gained by testing the strains of *E. coli* resistant to NG against structurally similar compounds.

Skinner *et al.*(3) have synthesized a series of nitrosoguanidines differing from NG in the substituent on N-1. In addition, 2 commercially available compounds, like NG, yield diazomethane under alkaline conditions. These are N-methyl-N-nitrosourea (MNU) and N - methyl - N - nitroso - *p* - toluenesulfonamide (MNTS). Another compound, S - methyl-N-nitropseudothiourea (MNPT) (7) will, like NG(8,9) nitroguanisate amino acids.

Methods. Strain S (obtained from Dr. A.

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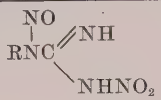
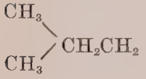
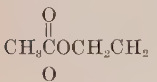
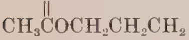
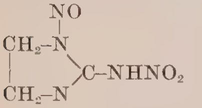
* This work was carried out under the auspices of the Cancer Chemotherapy National Service Center, Nat. Cancer Inst., N.I.H., U.S.P.H.S.

TABLE I. Effect of Various Nitrosoguanidines against *E. coli* Mutants Resistant to 1-Methyl-3-nitro-1-nitrosoguanidine. (Disc assay)



		Diameter of zone of inhibition (mm)*		
		E. coli strain		
R	μg/disc	S (parent)	S/Ng 1	S/Ng 2
CH_3 (NG)	25	23	10	0
CH_3CH_2	50	30	22	0
$CH_3CH_2CH_2$	50	30	27	10
$CH_3CH_2CH_2CH_2$	50	24	13	9
	20	18	7	2
	50	18	0	0
$CH_3CH_2CH_2CH_2CH_2$	50	12	0	0
Benzyl	20	14	3	0
 $CH_3\overset{\overset{O}{\parallel}}{C}OCH_2CH_2$	50	31	27	20
 $CH_3\overset{\overset{O}{\parallel}}{C}OCH_2CH_2CH_2$	50	28	13	±
$CH_3OCH_2CH_2$	50	29	14	11
$ClCH_2CH_2$	50	11	8	7
$ClCH_2CH_2CH_2$	50	31	27	20
	100	10	±	4

* In excess of disc diameter.

± = Barely visible zone of inhibition.

D. Hershey, Cold Spring Harbor, N. Y.) of *E. coli*, the parent, NG-sensitive strain, and the 2 mutants, S/Ng 1 and S/Ng 2, were used. The organisms were grown in the synthetic medium of Davis and Mingioli(10), pH 6.8, containing 1.5% agar, or in tryptone-agar, pH 5.5 (T-agar). T-agar is made as follows: 10 g of tryptone, 1 g glucose, 5 g sodium chloride, 12 g agar made up to one liter with distilled water and adjusted to pH 5.5 with hydrochloric acid.

Two methods of assay of drug activity were used: (1) Filter paper disc-assay. In this method, 10^7 log phase bacteria were spread on solid agar. Filter paper discs 0.67 mm in diameter were placed on the agar, and 20 μl of a properly diluted solution, usually methanolic, of test drug were added to the disc by micropipette. (2) The gradient plate technic of Szybalski and Bryson(11).

All cultures were incubated at 37°C for 18 hours, after which the zone of inhibition (disc-assay) or length of the streak showing confluent growth (gradient plates) was measured. Compounds were always tested concurrently on all 3 strains of *E. coli*.

Results. Table I shows results obtained with a series of nitrosoguanidines, varying in the substituent on N-1, against *E. coli* strain S, S/Ng 1, and S/Ng 2, grown on DM medium at pH 6.8. The last compound in the table (1-nitroso-5-nitraminoimidazoline) is included because it can be considered as a condensed nitrosoguanidine. Every compound in the table was less effective against S/Ng 1 than against S. With 4 of the compounds (R = propyl, acetoxyethyl, chloroethyl, and chloropropyl), the difference in zone size between S and S/Ng 1 was small and possibly within the error of the method. On the other hand, every compound was unequivocally less effective against S/Ng 2 than against S. Furthermore, wherever such a comparison is possible (zone size against S/Ng 1>0), and with only 2 exceptions, the compounds were less effective against S/Ng 2 than against S/Ng 1. The two exceptions were: R = chloroethyl and the nitrosoimidazoline. This last compound appeared to be less effective against S/Ng 1 than against S/Ng 2.

MNU and MNTS were inactive at concentrations up to 250 μg/disc when tested against *E. coli* strain S, DM medium, at pH 6.8. We had observed earlier that antibacterial activity of NG increased as pH of the medium at start of the experiment decreased. As measured by the effect on colony-forming ability of *E. coli* strain S, NG was over 1000 times as active in tryptone-agar at pH 5.5 (T-5.5 agar) as in DM medium at 6.8. When an amount of tryptone equivalent to that in T-5.5 agar was added to DM medium at pH 6.8, the activity of NG was significantly inhibited. It is possible that NG might be even more effective at pH 5.5 in absence of tryptone, but we have not yet been able to test this possibility, since *E. coli* did not grow at all in a salts-glucose medium at pH 5.5.

It was conceivable that MNO and MNTS

TABLE II. Effect of N-methyl-N-nitrosourea (MNU) and N-methyl-N-nitroso-*p*-toluenesulfonamide (MNTS) against *E. coli* in Tryptone-agar pH 5.5.

		Diameter of zone of inhibition (mm)*			
		Strain of *E. coli*			
Compound	μg/disc	S	S/Ng 1	S/Ng 2	S/Mnu 1
MNU	200	15	±	±	2
	100	21	±	0	2
	50	16			
	20	11			
	10	7			
MNTS	250	13	5	14	
NG	50		2	4	21
	25	23	10	0	15

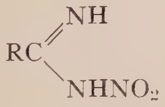
* In excess of disc diameter.

± = Barely visible zone of inhibition.

might inhibit *E. coli* on T-5.5 agar, even though inactive at pH 6.8. Both compounds produced zones of inhibition, even though at very high concentrations, under these conditions (Table II). Furthermore, S/Ng 1 was more resistant to both compounds than was S. However, S/Ng 2 was no more resistant to MNTS than was S. It was not possible to determine by this method whether S/Ng 2 was more or less resistant to MNU than was S/Ng 1. For this reason, we determined minimum inhibitory concentration (mic) of MNU on gradient plates (T-5.5 agar). The mic of MNU against *E. coli* strain S was 10 μg/ml; against S/Ng 1, 250 μg/ml; and against S/Ng 2, 165 μg/ml. As with MNTS and 1-nitroso-5-nitraminoimidazoline, S/Ng 2 was more sensitive to MNU than was S/Ng 1.

A mutant of *E. coli,* S/Mnu 1, was selected which was 25-fold resistant to MNU as determined on gradient plates. This strain was as resistant to NG as was S/Ng 1 (Table II).

Saroff and Evans(7) showed that MNPT nitroguanisated amino acids. This is a reaction characteristic of NG, and the two compounds resemble each other in having the structure



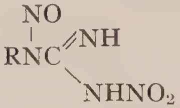
$$RC\begin{matrix}\diagup NH \\ \diagdown NHNO_2\end{matrix}$$

Since resistance to NG, at least in S/Ng 2, seemed to be a function of the nitroguanidine portion of the molecule, it seemed possible that NG and MNPT might be cross-resistant. However, MNPT was inactive against *E. coli* strain S at concentration of 100 μg/ml at pH's from 5.0 to 8.0.

Discussion. It is apparent from these results that resistance to NG is accompanied by resistance to all nitrosoguanidines, regardless of the alkyl substituent on N-1. At least this is true with the particular mutants we selected for resistance to NG. The demonstration of cross-resistance among nitrosoguanidines is unequivocal in S/Ng 2, and is questionable in only 4 of 11 compounds in S/Ng 1. In most instances it is clear that S/Ng 2 is more resistant to nitrosoguanidines than is S/Ng 1. It seems likely that if a more precise method for estimating degree of resistance were used, there would be no question about any of the compounds. Resistance of S/Ng 2 to NG and related compounds has previously been demonstrated clearly by conventional turbidimetric measurements(5). However, resistance to S/Ng 1 could not be demonstrated turbidimetrically; but where strain S responded to NG and related nitrosoguanidines by producing filaments, S/Ng 1 over an identical range of drug concentrations did not. Resistance of S/Ng 1 could be demonstrated only by examining the morphology of the organisms grown in liquid culture or by colony-forming ability on solid medium. The gradient plate technic might yield more precise quantitative data on relative degree of resistance among nitrosoguanidines. This method has been used by us with 3 different nitrosoguanidines, and the results, which substantiate the general conclusion regarding cross-resistance of any nitrosoguanidines, will be published later.

With the imidazoline derivative the results were different than with the nitrosoguanidines. Both S/Ng 1 and S/Ng 2 were resistant, but S/Ng 2 appeared to be less resistant than S/Ng 1. This was true also of MNU and MNTS: S/Ng 1 was resistant to both, and S/Ng 2 was no more resistant and was probably less resistant than was S/Ng 1.

How is this information related to what we already know? We know that S/Ng 1 is resistant to a variety of radiomimetic compounds (azaserine, Mitomycin C, nitrogen mustard) and to ultraviolet radiation (unpublished information). S/Ng 2 is no more resistant to these agents, and may be less resistant, than S/Ng 1. From this we conclude that the first step mutation, S/Ng 1, probably involves some general physiological change which makes the organism resistant to radiation and radiomimetic compounds. This change is associated with resistance to filament formation, or with some mechanism which permits the cell to divide properly or, on agar plates, to form colonies. In S/Ng 2, the second step mutation involves some mechanism which is concerned with the precise chemical configuration of NG. The problem is: how precise? It is obvious from the present data that S/Ng 2 shows increased resistance to compounds of the configuration



Therefore, the nature of the alkyl substituent on N-1 is of little or no importance in this stage of resistance. On the other hand, S/Ng 2 does discriminate between NG and MNU, or between a urea and a nitroguanidine, and between NG and MNTS. Resistance of S/Ng 2 is not against compounds which can yield diazomethane.

Since S/Ng 1 was resistant to MNU and MNTS, these compounds belong, in this respect, to a class of agents which include nitrogen mustard, azaserine, Mitomycin C, and which are radiomimetic. It is pertinent, therefore, that a mutant resistant to MNU was resistant to NG. This, again, we have observed with other compounds of this class: that first stage resistant mutants of each are cross-resistant with all other compounds of this class.

There are, therefore, at least two different types of events in the course of developing increased resistance to NG. One is a change in the physiology of the organism which involves a response to a variety of chemical compounds, all of which are radiomimetic, and to radiation itself. The other rejects the inhibitor *per se* or structurally related chemicals. With NG we have localized the portion of the molecule which seems to be concerned in the second type of resistance. We do not know, but may assume, that it is this portion of the molecule which is important to the inhibitory properties of the compound.

These experiments suggest many questions: Is the sequence of mutations—chemically nonspecific prior to chemically specific—inevitable? Or is the one more readily induced than the other? Or does the nonspecific mutation prime the organism for subsequent mutations which are chemically specific? Or does the specific mutation require a larger amount of inducer which is possible only in strains able to tolerate these amounts because of the nonspecific mutation? Do similar sequences occur in other radiomimetic agents? And, finally, do these events occur in vertebrate cells and could they account for resistance to radiomimetic agents in tumors?

Summary. A series of analogues of 1-methyl-3-nitro-1-nitrosoguanidines (NG), differing in the alkyl substituent, was tested against 2 mutants of *E. coli* strain S, selected for successively increasing resistance to NG. In general, increasing resistance to NG was observed to be accompanied by increasing resistance to all the nitrosoguanidines. When N-methyl-N-nitrosourea and N-methyl-N-nitroso - *p* - toluenesulfonamide were tested against NG-resistant strains of *E. coli,* it was found that the first step resistant mutant was cross-resistant. However, the second step mutant, derived from the first, was more sensitive to both of these compounds than was the first step mutant. A first step mutant of *E. coli* strain S, selected for resistance to N-methyl-N-nitrosourea, was resistant to NG. S-methyl-N-nitropseudothiourea did not inhibit *E. coli* strain S.

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Erythrocyte Survival in Vitamin E-Deficient Monkeys.* (26145)

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Anemia as the earliest sign of Vit. E deficiency in the monkey was first reported by Day and Dinning(1). The anemia was slightly macrocytic, and was accompanied by a granulocytosis as evidence of marrow activity. These findings suggested that the anemia occurring might be the result of an increased rate of erythrocyte destruction. Results of an experiment designed to test this hypothesis are the subject of this report.

Methods. Monkeys of the *rhesus* type which had been on a diet deficient in Vit. E for varying periods of time were selected for this study. The composition and preparation of the diet have been described(2).

Erythrocytes were tagged with radio-active chromium according to the principles set forth by Gray and Sterling(3) and Ebaugh *et al.*(4) and modified for application to small animals by Marvin and Lucy(5). The one departure from the technic as described was use of the brachial vein rather than cardiac puncture for obtaining blood for tagging, and use of the ear for obtaining repeated samples. Complete blood counts were made at intervals, also from the ear veins. Radioactive chromium (Cr^{51}) was determined in a scintillation well counter using a thallium-activated sodium iodide crystal.

Results and discussion. Survival curves of 6 studies on 4 monkeys are shown in Fig. 1. Monkey #211 served as a replete control, having never been given an E-deficient diet. Its erythrocyte count was 5.8 million per cu mm and hemoglobin and hematocrits were normal. Maximal survival time was 98 days; the curve was symmetrical with no apparent changes in rate of chromium loss. This normal value agrees well with that of 100 ± 20 days reported by Harne, *et al.*(6) using the consecutive reticulocyte peak method.

Monkey #204 had been on the deficient diet only 35 days when its cells were tagged and injected. On the 137th day of the dietary regimen, no activity could be detected; a maximal survival time of 102 days. During this time, erythrocyte count was 5.7-5.9 millions per cu mm. Monkey #205 had an 88 day survival time when measured between the 188th and the 276th day on the deficient diet. During this time red cell count decreased from 5.55 to 4.24 millions per cu mm. This is possibly not a significant, but certainly a suggestive, shortening of the life span.

On the other hand, after 349 or more days on the diet, survival was shortened to 49, 45, and 35 days in monkeys 207, 205, and 204, respectively. Erythrocyte counts were between 3.02 and 3.98 millions during the time these determinations were being made.

Five months after addition of adequate daily supplements of tocopherol were given monkey #205, red cell count had risen from 3.07 to 5.85 millions. Life span of the eryth-

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rocytes of this recovered monkey was 91 days (Fig. 2).

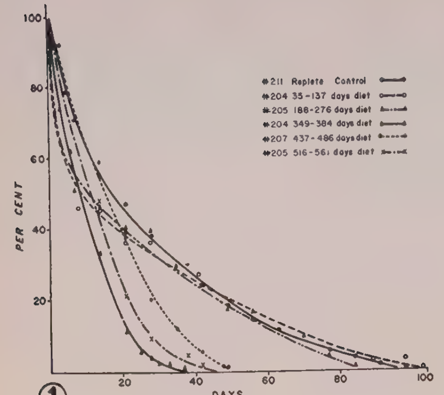
Curves in Fig. 1 show rather poor correlation between life span and time on the deficient diet. If, however, one plots lifespan against erythrocyte count, a respectable regression line can be drawn by inspection (Fig. 3).

The deficiency of Vit. E may alter the erythrocyte in such way that its longevity is impaired. The erythrocytes may thus die linearly due to a premature senescence. On the other hand, E-deficiency may result in a random destruction of erythrocytes regardless of age. A combination of these two mechanisms must certainly be considered. As pointed out by Sheets, *et al.*(7) removal of cells by a combination of random destruction and senescence can be represented by the formulation

$$N_t = N_o \left(1 - \frac{t}{T}\right) e^{-kt}$$

where N_o and N_t are numbers of cells initially and at time t, T is mean survival time and K is a constant characterizing rate of random destruction. With the chromium technic, K is a measure also of rate of elution. Rearrangement of the formula, solving for K(5) makes possible calculation of this constant. When values obtained are tabulated and inspected, it is apparent that rate of random loss of chromium (random destruction plus elution) persists longer at a higher level in the severely deficient monkeys than in those normal or only slightly deficient ones. This would substantiate the suggestion that the anemia of E-deficiency in the monkey is partly hemolytic(2).

The mechanism whereby this random "hemolytic" destruction takes place is of interest. György and Rose(8) reported that the erythrocyte of E-deficient rats could be hemolyzed in dialuric acid, but this agent did not appear to have a differential hemolytic effect on erythrocytes of premature infants (9). Such erythrocytes could, however, be hemolyzed by hydrogen peroxide(10), and this hemolysis was inversely proportional to plasma tocopherol levels(11). Horwitt *et al.* (12) showed that *in vitro* exposure of eryth-



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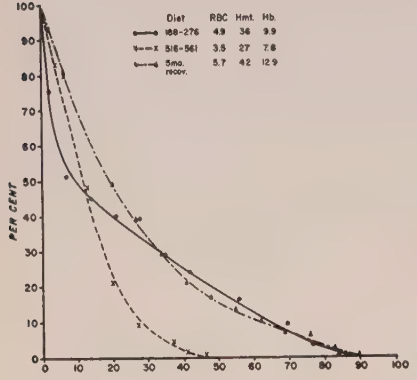

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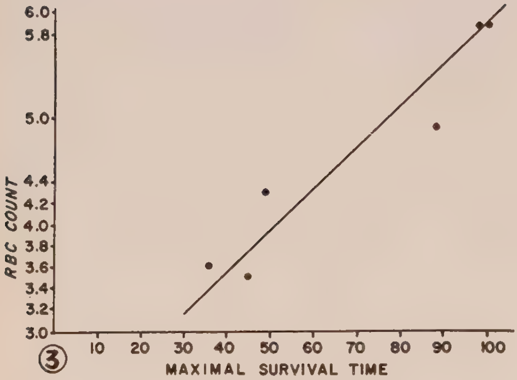

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FIG. 1. Curves showing disappearance of Cr^{51} from blood of 4 monkeys. Ordinate gives ratio of Cr^{51} content at various times after tagging to Cr^{51} content on day after tagging. Abscissa indicates time after tagging. Monkeys 204 and 205 were tested twice.

FIG. 2. Curves showing effects of vit. E-deficiency and replacement therapy on disappearance of Cr^{51} from the blood of Monkey #205. Coordinates the same as Fig. 1.

FIG. 3. Regression line relating maximal erythrocyte survival time on abscissa, and avg erythrocyte count during time of survival study on the ordinate.

rocyte stroma to hydrogen peroxide increased color production with 2-thiobarbituric acid; presumably reflecting an increased peroxide content. According to Deuel(13) peroxidation of unsaturated fatty acids at the ethene locus is followed by formation of hydroxy-keto-acids and finally cleavage of the chain. If this occurs in the lipid of the erythrocyte envelop and stroma, greater fragility and hemolysis might well result. The well documented antioxidant effect of alpha tocopherol (14) could prevent the action of hydrogen peroxide and other oxidizing agents *in vitro*. Oxidizing substances *in vivo* which could alter erythrocyte lipids in absence of Vit. E are not presently defined, but hydrogen peroxide as a metabolic transient might well be one of them.

It has also been shown that blood fat exerts a hemolytic effect(15) even at normal post-prandial levels(16). The diets used in our experiments, and generally used in studies of Vit. E deficiency, contained relatively large amounts of fat. Replacement with alpha tocopherol, without changing fat content of the diet, reduced the excessive rate of erythrocyte loss. It is possible, therefore, that Vit. E may interfere with hemolytic action of the ingested fat. If the binding of the fat by the erythrocyte is the critical point, as has been suggested(15), and this binding is oxidation-dependent, the antioxidant effect of Vit. E may be important. Further work is indicated before the mechanism is elucidated.

Summary. Survival of Cr^{51} tagged erythrocytes, autologously transfused, is severely shortened in monkeys maintained on Vit. E-deficient diets. Maximal survival times of 35, 45, and 49 days were obtained after monkeys had been on the diet for 349 or more days, as compared with about 100 days for replete controls. The shortened survival time, associated with anemia, could be reversed upon addition of Vit. E. The mechanism is discussed.

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Cytochemical Effects of Substituted Pyrimidines on Psittacosis Virus.* (26146)

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The metachromatic staining properties of acridine orange fluorochrome enables visual study of cytochemical changes which accompany certain viral infections(1). When stained with acridine orange and viewed by fluorescence microscopy, the fluorescence induced in the nucleoplasm of normal cells is yellow-green (DNA-staining) and in both cytoplasm and nucleoli is red-orange (RNA-staining)(2,3). Cytochemical changes associated with maturation of psittacosis virus§ in human cell lines have been described as a color sequence from shades of red to orange to yellow to green(4,5). Fluorescein-tagged antibody localized selectively in the mature virus inclusion. This host-parasite, differential-staining system offers a method for visual study of nucleic acid metabolism. It has been applied by us to studies on the mechanism of antibiotic action(6), and, as described herein, on the mode of action of fluorinated pyrimidines which are nucleic acid antagonists(7). The procedure is based on interference with the normal color sequence associated with virus maturation.

Methods. The procedures used for propagation and examination of tissue cultures infected with psittacosis virus have been described(5). Therefore, only a brief outline is given. A human synovial cell line (McCoy)(8) was propagated on coverslips in Leighton tubes. Tissue cultures were inoculated with a dose of virus (designated TTS) calculated to infect 99% of the cell population(9). Nutrient medium, without antibiotics, consisted of Mixture 199 with 2% heat-inactivated calf serum, and 0.5% lactalbumen hydrolysate. The effect of 5-fluorouracil (FU)‖ and of 5-fluoroorotic acid (FO)‖ on the maturation of psittacosis virus was tested. Graded concentrations (6-25 γ/ml) of FU and FO were each added at intervals from 0-24 hours to both infected and uninfected 3-day-old tissue cultures. After incubation at 37°C, coverslip preparations were fixed in acid-alcohol, stained with acridine orange, and examined by fluorescence microscopy(5). Fate of virus in treated and untreated cultures was followed by inoculation of whole culture extracts intracerebrally into mice and by the microscopic counting method(9).

Results. Within 14 hours after infection, the host-cell contributed an RNA-staining (red-orange) matrix around the initial DNA-staining (yellow-green) virus particle. The resulting cytoplasmic inclusion was designated the 14 hour "red-ball" stage (Fig. 1). RNAase treatment removed the matrix material and unmasked immature virus which was Feulgen positive, not antigenic to fluorescein-tagged antibody, and not infective for mice. During this period, the inclusion increased in size, number, and color intensity. The 14 hour "red-ball" inclusions were 1-4 μ in diameter. Within 38-72 hours, further development of the inclusion was noted. The RNA-staining matrix underwent a sequence of color changes from shades of red to orange to yellow to green (Fig. 2). Subsequently, pin-point mature virus appeared which was antigenically positive, DNAase-resistant, and infective for mice. Addition of FU prior to the 14 hour "red-ball" stage inhibited further color changes and hence virus maturation. In contrast, if FU was added after the 14 hour "red-ball" stage (during the period of matrix color change from red to orange),

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§ It is recognized that "virus" may be a misnomer.

‖ Courtesy of Dr. Robert Duschinsky, Hoffmann-La Roche, Inc.

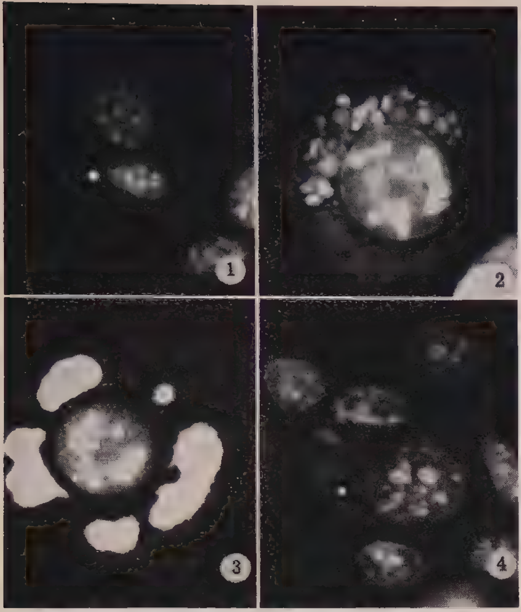

FIG. 1-4. McCoy cells infected with psittacosis virus. With acridine orange fluorochrome and ultraviolet microscopy, normal cytoplasm and nuclei fluoresce red-orange (RNA-staining) and yellow-green (DNA-staining), respectively. ×750.

FIG. 1. 14 hr "red-ball" stage. The cytoplasmic inclusion body which here appears white fluoresces a brilliant red-orange against a lighter cytoplasm.

FIG. 2. 48-72 hr after infection. Maturation of cytoplasmic inclusion bodies is indicated by a sequence of color changes from shades of red to orange to yellow to green which appear here as shades of white to grey. Subsequently, pin-point, DNA-staining mature virus is noted.

FIG. 3. 48 hr after addition of 25 γ 5-fluorouracil to the 14 hr "red-ball" stage (Fig. 1). Instead of the expected color changes indicated in Fig. 2, "fraudulent" RNA-staining inclusions are seen which here appear as white masses.

FIG. 4. 3 days after the stage shown in Fig. 3. The mass of "fraudulent" RNA-staining material disappears and unmasks an inclusion similar to the 14 hr "red-ball" stage.

some inclusion bodies showed evidence of maturation. Within 48 hours after addition of FU to the 14 hour "red-ball" stage, instead of the expected appearance of the yellow-green, DNA-staining mature virus, a mass of RNA-staining material was noted (Fig. 3). This abnormal, RNAase-sensitive, formation has been designated "fraudulent" RNA and may be analogous to that described by Bosch *et al.*(10). It was amorphous in appearance and distinct from both the lighter-stained uninfected cytoplasm and from the more brilliant 14 hour "red-ball" inclusion. Masses of "fraudulent" RNA disappeared gradually within 3-5 days in the course of which time the original 14 hour "red-ball" inclusion remained (Fig. 4). Uninfected control cultures exposed to similar concentrations of FU stained normally with acridine orange. Development of "fraudulent" RNA was more marked with FU than with FO. RNA-staining nucleoli of infected cells treated with FU were enlarged as were some cells. Psittacosis virus multiplied in cultures of McCoy cells; however, virus was absent from infected cultures in which "fraudulent RNA" appeared following treatment with at least 6 γ FU per ml nutrient fluid. This inhibition was partially reversed when 20 times the amount of uracil was added to the FU (Table I).

Discussion. Viruses have been defined as transmissible genetic material(11). As such, they induce changes in infected cells which result ultimately in replication of the original agent. The normal course of psittacosis virus infection of human cell lines showed that the initial DNA-staining virus triggered a reaction in which a matrix of RNA-staining material was formed. That this matrix material gradually faded and disappeared coincident with the appearance of mature virus suggested that it functioned as a metabolic pool or source of nutrients and energy for replication of virus. The matrix material appeared to be involved in production of viral protein-coat and in production of viral DNA itself. These observations are in accord with the roles designated for RNA(12,13). There are indications, also, that FO and FU interfere with DNA formation but not with RNA or protein synthesis(14). In our studies, FO

TABLE I. Effect of 5-Fluorouracil on Replication of Psittacosis Virus in McCoy Cells.

		Virus recovered 4 days later	
Drug added	γ/ml	Mouse inoculation	Infective units/ml
FU	6	0/5*	20†
FU + uracil	6 + 120	2/5	1.1×10^3
None	—	5/5	1.1×10^7

* No. infected/No. inoculated.
† Particle count in tissue culture.

and FU interfered with the DNA (virus) to RNA (intermediate) to DNA (virus) sequence. Instead of the expected cycle, these pyrimidines caused accumulations of abnormal RNA-staining material which inhibited virus maturation. Removal of this "fraudulent" RNA-staining matrix with RNAase unmasked DNA-staining immature virus. These observations suggested that FU and FO did not interfere with RNA or DNA synthesis *per se* but did interfere with development of intact virus through inhibition of protein synthesis.

When chlortetracycline HCl was added to the nutrient medium at the "red-ball stage" of psittacosis virus, maturation of the inclusion was arrested and virus was not recoverable from such cultures(6). While chlortetracycline and FU interfered with virus replication, the mechanism of each was different: the former appeared to block the mobilization of an RNA-staining matrix, while the latter interfered with the normal biosynthetic sequence by formation of an abnormal intermediate compound.

Summary. Cytochemical changes associated with psittacosis virus infection of tissue cultures were studied by acridine orange staining and fluorescence microscopy. Normal virus development was interrupted by the addition of 5-fluorouracil and 5-fluoroorotic acid which caused the accumulation of "fraudulent" RNA. It is suggested that the substituted pyrimidines interfered with development of intact virus through inhibition of protein synthesis.

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Permeability of Ehrlich Ascites Cells to Adenine.* (26147)

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Data on the permeability of cell membranes to a considerable number of non-electrolytes are available(1,2). Relatively little is known of the permeabilities of cells to the purines and pyrimidines although nucleic acid metabolism has been a major area of investigation for over a decade.

The present paper reports our work on permeability of Ehrlich ascites cells to adenine.

Methods and materials. The Ehrlich ascites used was a hypotetraploid line carried in Swiss albino mice by weekly intraperitoneal injections of 0.1 ml of ascites. For experiments, 6 day ascites was collected into 50 ml Krebs-Ringer bicarbonate (KRB) containing 0.2 mg heparin. The resulting suspension was diluted with an equal volume of

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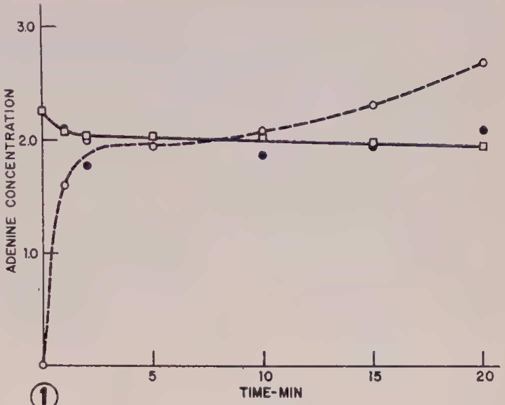

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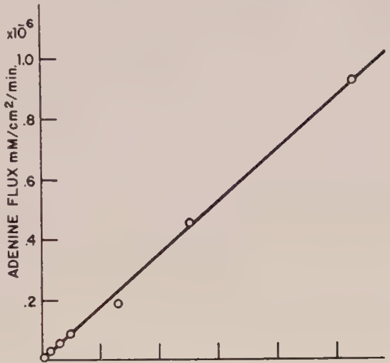

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FIG. 1. Kinetics of penetration of adenine into Ehrlich ascites cells. –□– c_e in mM/l; –○– c_i in mM/kg H_2O, calculated from change in c_e; –●– c_i calculated from adenine found in pellets.

FIG. 2. Dependence of adenine flux on avg extracellular concentrations.

distilled water to hemolyze the contaminating erythrocytes and centrifuged to collect the cells. After one wash with KRB, the cells were resuspended in KRB. Suspensions which had not been subjected to osmotic hemolysis were used in some experiments. The two types of preparations did not differ in adenine permeability.

The permeability constant was determined at 4°C and at 37°C. All procedures were carried out in a cold room at 4°C except for the incubations at 37°C. For the determinations at 4°C, a cell suspension and a solution of adenine in KRB were mixed and at various times aliquots were taken and centrifuged at top speed in a Phillips-Drucker 703 centrifuge. Time of contact between cells and adenine was taken to be the time from mixing until the centrifuge reached top speed. For the studies at 37°C, the cell suspension and adenine were put into separate arms of a Heinicke reaction vessel which was then gassed with 5% carbon dioxide in air and stoppered. After a preincubation of 4-5 minutes at 37°C, the contents of the 2 arms were mixed. At the end of the incubation, the vessels were chilled rapidly in ice water, taken to the cold room and samples were centrifuged to separate cells from the suspending medium.

The supernatant fluids were extracted by adding 4 ml of 95% ethanol to one ml of supernatant and centrifuging. The resulting precipitates were washed with 5 ml of 75% ethanol containing .09% NaCl. The extract and wash were combined and diluted to an appropriate volume with 75% ethanol. The adenine was determined spectrophotometrically(3).

In some experiments the cell pellets were extracted with 75% ethanol containing .09% NaCl, the extracts were dried, dissolved in water and spotted on Whatman No. 1 paper for chromatography. Ascending paper chromatograms were run in a solvent consisting of isoamyl alcohol—5% Na_2HPO_4 (9:21).

On all samples, cytocrits, dry weight of cells and total water in pellets from 2 ml of suspension and cell counts were determined. The volume of extracellular fluid trapped in the pellets was read from a calibration curve of the sucrose space of pellets of Ehrlich ascites cells obtained with our standard technic(4).

The amount of adenine entering the cells was determined by difference from the drop in extracellular concentration during the incubations. In some experiments the total pellet adenine was determined from paper chromatograms of the pellet extracts.

Results and discussion. Fig. 1 shows the kinetics of entrance of adenine into Ehrlich ascites cells at 37°C. Within a few minutes the *calculated* intracellular concentration plateaus at a value slightly below the extracellular but then rises to exceed the extracellular by 20 minutes. Since intracellular concentration is calculated from the amount of adenine disappearing from the extracellular fluid this might be explained by a slow metabolism of adenine. However, the adenine in the pellets was also determined directly by paper chromatography of the pellet extracts, elution of the adenine spots with ammonia water (pH 10.5) and determination of adenine spectrophotometrically. The adenine found in the one and 5 minute pellet samples was unaccountably high. There was an increase in adenine recovered in the pellets from 10 to 20 minutes suggesting that the increase in the calculated intracellular concentration might be partly a true increase and not entirely due to a metabolic conversion of the adenine. This might be explained if the adenine slowly accumulated in some compartment of the cell, perhaps one with a partition coefficient against water greater than one for adenine. The data in Fig. 1 suggest that adenine enters the cell by a simple diffusion mechanism. Further evidence for this was obtained by measuring the amount of adenine entering the cells in the first 2 minutes of incubation at various extracellular concentrations of adenine. Fig. 2 shows that the flux entering the cells is a linear function of average extracellular concentration. This is the expected result if only a simple diffusion is involved in this process. This finding makes it unlikely that any carrier or active transport system is involved in the entrance of adenine into Ehrlich ascites cells, although it does not unequivocally rule these out. The slope of this line gives a kp of $1.9 \cdot 10^{-4}$ cm/min at 37°C.

Permeability constants were determined by measuring the fall in extracellular concentration in the first 10 minutes at 4°C and in the first 2 minutes at 37°C. The extracellular concentration changed less than 15% in these experiments; usually this change was less than 10%. Thus it was assumed that intracellular concentration was given by $c_i = (c_e)\left[1 - e\,\frac{-kpA}{Vi}\,t\right]$ where kp is permeability constant, A/Vi is ratio of cell surface area to intracellular water volume, and (c_e) is the average extracellular concentration during the period involved. Intracellular concentration was determined by difference from the change in extracellular concentration. Table I gives the results. The kinetics of adenine uptake in the first 5 minutes shown in Fig. 1 would fit a kp of about 2.10^{-4} cm/min. The data in Table I may be expected to have an error of as much as 50% because of the inherent difficulty in measuring a small quantity by difference between two large quantities. The data do show that the kp at 37°C is in the range 10^{-4} to 2.10^{-4} cm/min and that it is about 5 fold greater at 37°C than at 4°C.

TABLE I. Permeability of Ehrlich Ascites Cells to Adenine.

Temp., °C	kp, cm/min.	Temp., °C	kp, cm/min.
4	$2.3 \cdot 10^{-5}$	37	$.6 \cdot 10^{-4}$
4	$1.9 \cdot 10^{-5}$	37	$1.9 \cdot 10^{-4}$
4	$2.6 \cdot 10^{-5}$	37	$1.0 \cdot 10^{-4}$*
37	$1.0 \cdot 10^{-4}$	37	$.6 \cdot 10^{-4}$†
37	$1.0 \cdot 10^{-4}$	37	$.8 \cdot 10^{-4}$

* In presence of glucose at 100 mg %.
† *Idem* at 200 " .

Summary. Adenine enters Ehrlich ascites cells by simple permeability. The permeability constant for adenine is in the range 10^{-4} to 2.10^{-4} cm/min at 37°C and 2.10^{-5} cm/min at 4°C.

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Effect of Deuterium Oxide on Growth of HeLa, L and L-5178Y Cells.*†
(26148)

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Many studies have been carried out on the effects of deuterium oxide on growth of plants, bacteria, protozoa and animals(1). In general, these studies have shown that deuterium in high concentrations is toxic in some degree to all living organisms. As the organism under study becomes more highly organized deuterium becomes more toxic, thus algae can grow in 99% D_2O(2), while mice cannot survive 40% deuteration(3). The toxic effect of high concentrations of deuterium oxide on cells grown in tissue culture was first observed by Fischer, working with chick fibroblasts, a mouse carcinoma and Rous sarcoma(4). Cagianut(5) commented on certain aspects of the cytopathology of tissue cultures of rabbit connective tissue and recently Siegel *et al.*(6) have studied the cytopathology of HeLa cells grown in D_2O. The common observations have been cell clumping, cell enlargement, multiple nuclei, nuclear pyknosis and vacuolation. Some of these characteristics have also been seen in D_2O-grown *E. coli*(7). This report deals with effects of D_2O on growth and cytological characteristics of 3 lines of tissue culture cells.

Results and discussion. Three cell lines of diverse origins were used: HeLa, a stable epithelial line of human origin; L cells, a murine fibroblast strain; and L-5178Y, a stable cell line derived from a spontaneously occurring murine leukemia of thymic origin. HeLa and L cells are usually grown as a monolayer on the surface of glass containers; they generally exhibit mass doubling times of 24-48 hours. L-5178Y is a round cell which does not adhere to glass and therefore can be grown in suspension as either a stationary or agitated culture(8). It is unique in that it shows an 11.5-hour generation time at 36°C during logarithmic growth.

TABLE I. Effect of D_2O on Yield of HeLa and L Cells.

% D_2O in growth medium	Total yield of cells* HeLa	L
0	100	100
10	87	85
20	64	54
30	49	34
40	23	16
50	13	4

* As % of control.

The studies with HeLa and L cells were carried out in Leighton tubes. Freshly trypsinized cells (150,000) were planted per tube in 1 ml of Eagle's basal medium, 10% in calf serum, containing varying amounts of heavy water. The medium in each tube was changed on alternate days. After 8-10 days at 36°C the cells were harvested with trypsin or trypsin-versene and cell yields determined (Table I). Cell counts were determined by means of hemocytometers. Each count reported is the mean of 4 replicate determinations and shows a statistical variation of ± 10%. It can be seen that 10% heavy water has little effect on growth rate, whereas 40 and 50% heavy water is toxic. The final number of cells recovered at these D_2O concentrations (40 and 50%) was less than the original inoculum.

The influence of D_2O on mass doubling time of logarithmically growing L-5178Y is shown in Table II. With this rapidly growing cell, an effect can be seen even at 5% D_2O. At 30% D_2O, the mass doubling time is almost twice the normal value. At all levels of D_2O below 40% the cells grew logarithmically for at least several weeks. The value found in 50% D_2O can be determined only

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† We would like to thank Dr. G. A. Fischer for his generous gift of L-5178Y and his help in establishing this cell line in our laboratory.

TABLE II. Effect of D_2O on Mass Doubling Time, Wet Weight and Dry Weight of L-5178Y Cells.

D_2O in growth medium (%)	Mass doubling time (hr)	Wet wt	Dry wt
		(mg/10^6 cells)	
0	11.5	.71	.12
5	12.3	.75	.12
10	13.0	1.20	.15
20	14.2	1.20	.18
30	19.0	1.50	.15
40	29.0	1.50	.15
50	56.0	—	

over the first 96 hours after exposure of the cells to this medium. During this time the cells grew; then cell counts began to decrease and the culture died. This leukemia was grown in the medium devised by Fischer(8).

A more detailed investigation was made of the effect of D_2O on L-5178Y and HeLa cells. In Table II are shown wet weight and dry weight of L-5178Y cells raised in media of varying D_2O concentration. There is a distinct increase in water content of the cells as D_2O concentration in the growth medium is increased. There is also a slight increase in dry weight of the cells.

HeLa cells were exposed to a medium containing 20% heavy water for varying lengths of time, and the wet weight and dry weight of the cells determined (Table III). Again we see a slight increase in both dry weight and water content of the cells. The composition of these deuterated cells with respect to proteins, nucleic acids, polysaccharides and lipids is now under investigation.

Cytological observations. The morphological and cytochemical changes induced by D_2O in 2 cellular systems *in vitro,* HeLa and L cells, are essentially similar. Briefly, in both cell types one can observe a progressive increase in number of large polynucleated cells paralleling the increase in concentration of D_2O in the medium. This effect is more evident with the HeLa line, in which multinucleated cells are present in only 1-2% of the normal cell population; thus even in 10% D_2O the number of multinucleated cells is increased, and at 40% D_2O, between 30-35% of the cell population is made up of large cells with 2-3 nuclei, and occasionally more, with an enormous cytoplasm (Fig. 1-4).

TABLE III. Effect of 20% D_2O on Wet and Dry Weight of HeLa Cells.

Time of exposure (days)	Control (H_2O)*		D_2O*	
	Wet wt	Dry wt	Wet wt	Dry wt
3	5.7	.65	5.2	.70
6	5.5	.70	6.5	.80

* Values are mg/10^6 cells. Weights given are avg of 4 determinations and show a variation of less than 2%. They are subject to the statistical limitations of the cell counting method.

The cytoplasm appears as an amorphous protoplasmic mass, and even with phase contrast, very few mitochondria can be discerned; the Golgi areas seem to be hypertrophic. The nuclei are slightly enlarged; no particular variations in size and number of the nucleoli has been observed. The general cytological characteristics of the deuterated cells are, then, very similar to those of the giant cells produced by irradiation(9). In the case of L cells, enlarged and polynucleated forms are also present, but not to the striking degree observed with HeLa cells; in addition, L cells in 20% D_2O lose their characteristic cytoplasmic filament, the cell border becoming poorly defined. No significant changes in regard to DNA and RNA were demonstrable by Feulgen and pyronin staining. In only one experiment with L cells was there a striking loss of cytoplasmic RNA, unaccompanied by changes in the nucleolar RNA. Lipid stains (Sudan black and Nile blue sulphate) have given equivocal results; in general, we observed a moderate increase of sudanophilic material and a shift in the morphology of this material in the sense that the small, uniform granules—lipochondria—which are present in the normal cells. decrease, while large, irregular masses of sudanophilic material increase. This increase has not been uniform in various experiments and seems to be more evident with L cells. In one HeLa experiment, there was a suggestion of an increase in neutral fat, as demonstrated by Nile blue sulphate. It is probable that the extent of lipid changes is related to the general condition of the cells at time of deuteration, and possibly to the length of time they are exposed to heavy water.

Summary. The effect of D_2O on growth of 3 stable mammalian cell lines (HeLa, L

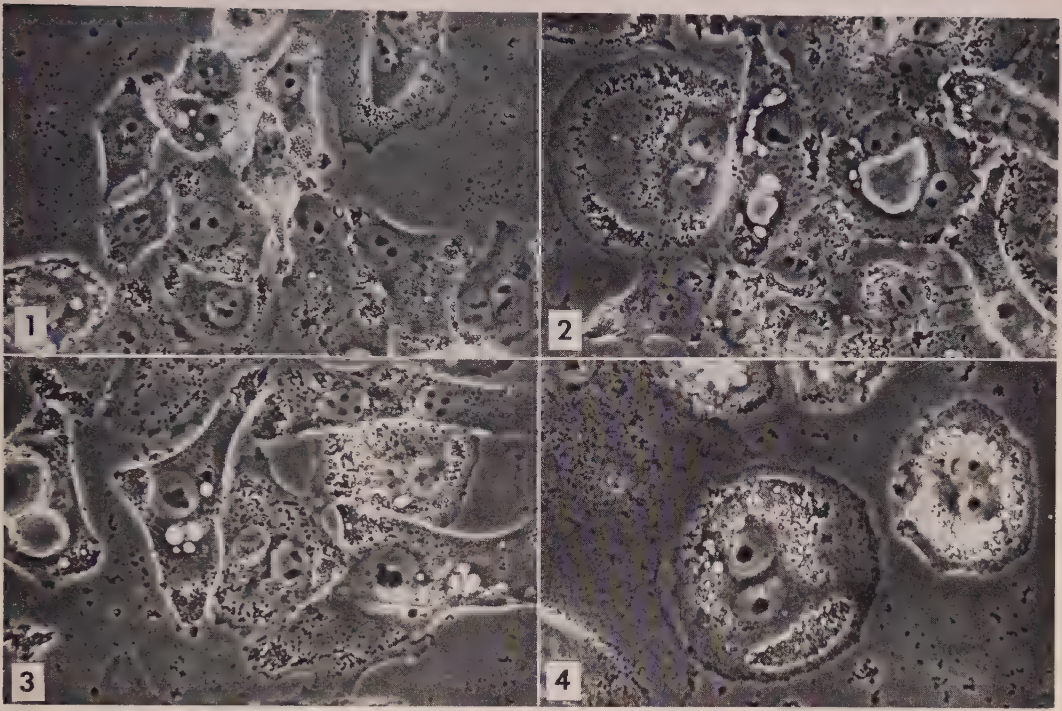

FIG. 1. HeLa cells in H_2O-containing medium. Cytoplasmic and nuclear characteristics are evident. Phase contrast × 293.

FIG. 2. HeLa cells in 20% D_2O-containing medium. This field is occupied by a large binucleated cell and by mononucleated cells which show an increased size. In addition, conglomerates of refractile granules (lipochondria) are visible at periphery of cytoplasm. Phase contrast × 293.

FIG. 3. HeLa cells in 30% D_2O-containing medium. All cells are enlarged and several are binucleated. Several cells have large cytoplasmic vacuoli which are sudanophilic in stained preparations. Phase contrast × 293.

FIG. 4. HeLa cells in 40% D_2O-containing medium. There has been a decrease in cell number and all residual cells show a higher degree of the changes illustrated in previous pictures. In this field 2 enlarged binucleated cells are seen together with a large portion of cytoplasm of another giant cell. Phase contrast × 293.

and L-5178Y) has been investigated. As D_2O concentration was increased all cells showed increased water content and dry weight and a slower growth rate. Cytological investigations showed an increase in number of multinucleated cells and a moderate increase in sudanophilic material. No significant changes in nucleic acid content were demonstrable with the staining technics used.

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Porphyrin Metabolism in Mice Following Quantitative Administration of Collidine Compounds and Oxidation Products. (26149)

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In a study involving the fluorescence of the forestomach of mice, diethyl-1, 4-dihydro-2, 4,6 - trimethylpyridine - 3, 5 - dicarboxylate (hereafter called "collidine derivative") was added to a white bread diet for the mice(1). The "collidine derivative" was blue fluorescent but it caused the gall bladder and part of the intestines to become bright red fluorescent. It was concluded that the "collidine derivative" probably disturbed the porphyrin metabolism of the mice to such a degree that the bile became red fluorescent(1). This hypothesis was later tested and validated(2). It was thus established that the "collidine derivative," when fed to mice, produced a disturbance in porphyrin metabolism characterized by marked increases in hepatic proto- and coproporphyrin. The oral method of administration made a precise and uniform dosage of the agent impossible. In the present study, the "collidine derivative" was injected intraperitoneally daily to achieve a more accurate method of drug administration. In addition, the effects of chemical alteration of the "collidine derivative" molecule have been studied and the hepatic coproporphyrins were subjected to isomer analysis.

Materials and methods. Twenty-five male mice of the ZBC strain weighing 20-25 g were housed in the dark and exposed to tungsten light only during the time of the daily injection. "Collidine derivative" dissolved in Mazola corn oil 20 mg/ml was injected but it was necessary to warm the solution slowly before use to dissolve completely the "collidine derivative." Daily injections of 0.25 ml/mouse were administered intraperitoneally. Liver samples were prepared as described previously(2) and subjected to porphyrin analysis using the method of Schwartz and Wikoff(3). Qualitative analysis for green porphyrins was carried out according to the method of Schwartz and Ikeda(4). Qualitative isomer determinations of hepatic coproporphyrins were made employing ascending paper chromatography (method of Dr. Mohammed Azziz, to be published).

The following compounds were prepared according to the method of Hantzch(5): 1) diethyl-2,4,6-trimethylpyridine-3,5-dicarboxylate. 2) 2,4,6-trimethylpyridine-3, 5-dicarboxylic acid. 3) Dipotassium-2,4,6-trimethylpyridine-3,5-dicarboxylate. Since the first compound is an oil, it was tested by intraperitoneal injection. The remaining 2 compounds were added to a ground food diet (1:18). Thirty male mice of the A strain weighing 20-25 g were used to test these substances.

Results. Animals receiving "collidine derivative" *via* the intraperitoneal route showed a disturbance in porphyrin metabolism similar to those receiving the drug in their diets (2). Hepatic protoporphyrin was present in much higher concentration than hepatic coproporphyrin. No green porphyrins could be detected in livers of animals treated for 10 days. Isomer analysis revealed the presence of only coproporphyrin III in hepatic tissue of animals receiving "collidine derivative" for 10 days. Examination of livers from animals treated with diethyl-2,4,6-trimethylpyridine-3,5-dicarboxylate; 2,4,6-trimethylpyridine-3,5-dicarboxylic acid; and dipotassium-2,4,6-trimethylpyridine-3,5-dicarboxylate revealed no increases in hepatic porphyrin concentrations within a 10-day period of treatment.

Discussion. The disturbance in porphyrin metabolism caused by "collidine derivative" has been contrasted with Sedormid porphyria (2). Both agents cause increases in hepatic proto- and coproporphyrin. The high concentrations of urinary porphobilinogen and uroporphyrin in Sedormid porphyria are not found following administration of "collidine derivative." Large quantities of green por-

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phyrins were noted in hepatic tissue of Sedormid-treated animals by Schwartz and Ikeda (4). Accumulation of these compounds in Sedormid porphyria and their absence in porphyrin induced by "collidine derivative" is further evidence of dissimilar mechanisms of the action of these two agents. Examination of sections of mouse liver (stained with H & E) from animals receiving "collidine derivative" for 7 days revealed a marked proliferation of bile ducts and periportal fibrosis. The portal areas were infiltrated with inflammatory cells. This picture of liver injury was associated with increased hepatic coproporphyrin III levels. No coproporphyrin I could be detected in livers of treated animals.

Mild oxidation of "collidine derivative" yields diethyl-2,4,6-trimethylpyridine-3,5-dicarboxylate. The latter compound has no effect on the porphyrin content of mouse liver. Williams(6) states that pyridine is oxidized *in vivo* to methylpyridinium hydroxide and excreted as an ethereal sulphate and a conjugated glucuronide. Similarly, picoline is oxidized *in vivo* to picolinic acid and excreted as the glycine conjugate, pyridinuric acid. The metabolism of "collidine derivative" has not been described; however, its activity may be related to an inability of liver to convert it to the oxidized, inactive compound: diethyl-2,4,6-trimethylpyridine-3,5-dicarboxylate.

Conclusions. Parenteral administration of 5 mg/mouse/day of diethyl-1, 4-dihydro-2, 4,6-trimethylpyridine-3,5-dicarboxylate ("collidine derivative") in corn oil causes increased hepatic and biliary proto- and coproporphyrin levels. Hepatic coproporphyrin was found to be entirely Type III isomer. No green porphyrins are found in livers of animals receiving "collidine derivative." Oxidation products of "collidine derivative" did not produce abnormalities in porphyrin metabolism within a 10-day observation period.

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Glucose-6-Phosphatase Activity in Various Sheep Tissues.* (26150)

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The significance of carbohydrate in ruminant metabolism differs in some ways from that in other mammals(1). Of special interest are the chemical precursors and sites of formation of glucose, because little glucose appears to be absorbed from the ruminal digestive tract(1). Arteriovenous differences have suggested that glucose may be contributed to the blood of sheep not only by the liver but also by extra-hepatic tissues(2). A similar result on ruminant-like marsupials has been reported(3). It is usually considered that tissues which can release glucose into the blood have glucose-6-phosphatase activity, and in the rat this is largely restricted to liver, kidney and intestine(4). To investigate the possibility that ruminants differ in this respect from rats we have estimated the glucose-6-phosphatase activity of various sheep tissues.

Methods. The technic has been described by Hers and de Duve(4,5). We repeated their work on rats, then continued with adult sheep. About 5 g samples of liver, kidney, intestine, salivary gland, brain, muscle, rumen epithelium and mammary gland were

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TABLE I. Glucose-6-Phosphatase Activity of Tissue Homogenates Expressed as mg P Liberated per Gram of Tissue from 4.2 × 10^{-3}M G-6-P at pH 6.5 and 37°C for 10 Min.

Tissue	Rat*	Sheep†
Liver	2.08	1.37 ± .16 (7)
Kidney	1.47	1.60 ± .20 (7)
Intestinal wall	1.08	.13 ± .05 (5)
" mucosa	—	.21 ± .10 (3)
Salivary gland	—	.10 ± .02 (9)
Muscle	trace	trace
Brain	—	"
Rumen epithelium	—	"
Mammary gland	—	"

* Mean of 2 observations.

† Mean ± stand. error (No. of observations).

taken from sheep killed by cutting the carotid and jugular vessels. Aqueous homogenates were prepared in the cold, the supernatants were incubated with glucose-6-phosphate (G-6-P), protein was precipitated with trichloracetic acid, and phosphorus in the filtrates was estimated by the Fiske and Subbarow method (for details see 4,5).

Results are shown in Table I. On the whole our results with rat tissues confirmed those of Hers and de Duve(4), although we found rather less glucose-6-phosphatase activity in the liver and more in kidney and intestine, but observed the same order between organs, liver having the highest activity. The glucose-6-phosphatase activity of ovine intestinal wall and mucosa was much smaller than that of kidney and liver. It was also smaller than the activity of intestinal wall tissue from the rat. The enzyme activity was detectable also in salivary gland homogenates but was negligible in the other tissues.

Discussion. The low glucose-6-phosphatase activity of sheep intestine is in accord with other types of observations (recently reviewed by Lindsay)(1), which suggest that little glucose enters the portal blood from the digestive tract of sheep. The high phosphatase activity of ovine kidney suggests that the renal contribution of glucose to the blood, which has been estimated at 4-13% of the hepatic contribution in the dog(6), may be even greater in sheep. The slight phosphatase activity in the salivary gland leaves open the possibility that a little glucose might also be added to the blood by these glands (cp. 2).

Summary. Glucose-6-phosphatase activity was estimated in various sheep tissues. It was appreciable in liver and kidney, detectable in intestine and salivary gland, but negligible in brain, muscle, stomach, rumen epithelium and mammary gland. The results suggest the relative importance of these tissues in contributing glucose to the blood in sheep. They indicate particularly the relatively small potential role of sheep intestinal wall as a source of blood glucose.

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A Biological Assay for Ribonuclease with Sub-millimicrogram Sensitivity
(26151)

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Ribonuclease (RNase) activity isolated from different sources can be assayed by chemical(1,2), manometric(3), spectrophotometric(4), turbidimetric(5), and isotopic(6) methods. The substrate in these methods is usually yeast ribonucleic acid (RNA) and measurements are made of its disappearance or hydrolysis products.

Recommended enzyme levels for most of these assays range from 5 to 50 μg of enzyme in 0.1 to 1.0 ml volumes, although modifications can yield greater sensitivity particularly with the isotopic method.

The use of biologically active RNA as an ultra-sensitive substrate for determining RNase was suggested by the observation(7) that a commercial preparation of crystalline deoxyribonuclease (DNase) (Worthington Biochem. Corp.) inactivated infectious RNA derived from foot-and-mouth disease virus (FMDV). It was presumed and has now been proved(8) that such action was caused by RNase impurities in the DNase which are undetectable by conventional measurements.

This communication reports a biological assay for RNase activity capable of detecting this enzyme at concentrations as low as 0.1 mμg per ml. Infectious RNA derived from FMDV was used as the enzyme substrate and primary cell cultures of calf kidney as the RNA substrate.

Materials and methods. Infectious FMDV ribonucleic acid was isolated from purified preparations of tissue culture FMDV, type A, strain 119, by the phenol extraction procedure of Gierer and Schramm(9), as adapted to FMDV in our laboratory(7). The RNA preparations, ranging in activity from 10^3 to 10^4 plaque-forming units per ml (PFU/ml), were diluted with Hanks' balanced-salt solution containing 0.5% lactalbumin hydrolyzate. *RNA plaque assay* was conducted in 4-oz prescription bottles containing primary cell cultures of calf kidney. The cell layers were triply washed before use with Hanks' balanced-salt solution containing 0.5% bovine lactalbumin hydrolyzate to remove serum. The RNA preparations were then applied. After 15 minutes at room temperature, the cultures were overlaid with agar medium and incubated 72 hours at 37°C as described elsewhere(7). Plaques were then counted and expressed as PFU/ml. *Ribonuclease* was obtained from Worthington Corp. as a crystalline preparation.

Experimental and results. Calibration for the procedure was as follows: Using 16 x 150 mm test tubes, 0.3 ml portions of infectious RNA containing from 500 to 5000 PFU/ml were mixed by repeated pipetting with 0.3 ml portions of RNase suitably diluted in 0.02 M, pH 7.5 phosphate buffer. After 15 minutes in a water bath at 25°C, with occasional shaking, the tubes were cooled in an ice bath, and the contents assayed for RNA plaque-forming units. Table I shows typical results.

TABLE I. Calibration of RNase Biological Assay.

Dilution of RNase*	PFU/ml of enzyme treated RNA
1:2500	0
1:5000	40
1:10,000	180
1:20,000	520
Control RNA = 500 PFU/ml	

* 1 μg/ml RNase.

The results are expressed directly in terms of weight of the enzyme present, rather than as units derived from a rate determination. The end point is represented by the highest dilution of enzyme preparation which still showed activity against the infectious RNA used as substrate. As little as 0.1 mμg of RNase can be detected by this assay method. A 2-fold dilution series was used for the final determination, although 10- or 100-fold dilution series may be needed for the preliminary investigation of an unknown sample. Less than 2-fold dilutions are not practical for final determination of enzyme activity, because of the known limitations in the precision of the plaque assay for infectious RNA (7). That is, the range in number of plaques found in equal-sized samples from a single RNA population is m ± 25% when mean value, m, is about 40 plaques.

A study of the effect of time and temperature on the reaction between enzyme and substrate appears in Table II. No enzyme activity was detected at a 1:2500 dilution of 1 μg/ml RNase acting for 1 hour at 4°C, although tests had shown considerable activity with RNase at a concentration of 5 μg/ml (7). Lengthening the exposure time at 25°C produced greater sensitivity, but the 4 hours needed for maximal activity was not required for routine assays. There was no benefit in using temperatures higher than 25°C, and sensitivity even seemed to be lost.

To determine that the reaction being

TABLE II. Effect of Time and Temperature of Incubation between RNase and FMDV-RNA.

Min.	Temp. (°C)	% of control activity*
5	4	100†
15	"	100†
60	"	100†
15	25	30
30	"	35
60	"	20
120	"	15
240	"	0‡
5	37	30
5	60	65

* 1:10,000 dilution of 1 μg/ml RNase used.

† There was also 100% of control RNA activity (therefore no enzyme activity) for 1:2500 enzyme dilution.

‡ When enzyme was diluted further to 1:20,000, 20% of control RNA activity remained.

studied represented a hydrolytic breakdown of the RNA substrate and not formation of an inactive RNase-RNA complex, an RNA preparation (3,000 PFU/ml), which had been inactivated at 25°C with a 1:500 dilution of a 1 μg/ml RNase solution, was re-extracted with phenol in the usual way, and the protein-free RNA preparation then assayed. There was no infectious activity in the enzyme-treated RNA sample, while a control RNA sample re-extracted with phenol retained practically its full activity.

The RNase assay described was applied to the RNase content of sera from various sources (Table III). Except for guinea pig serum, values for the human and animal sera fall within a narrow range, 0.02-0.12 μg/ml. Since the guinea pig serum had a 20- to 30-fold greater activity, it was investigated further to determine whether its increased activity was entirely due to RNase action.

The possible presence of a low-molecular-weight RNA inhibitor or cofactor was investigated. Guinea pig serum was diluted 1:10 in 0.02 M phosphate buffer of pH 7.5 and dialyzed against 1000 volumes of the same buffer for 24 hours at 4°C; the buffer liquid was changed once during this interval. The dialyzed guinea pig serum as well as undialyzed serum were assayed against FMDV-RNA, and no significant difference was found in their RNase activity. Similarly, horse serum, used as an example of a low enzyme content serum, did not change significantly in activity upon dialysis.

Tests were made for presence of acid- or heat-labile RNA inactivators. Guinea pig serum, diluted 1:10 in phosphate buffer and adjusted to pH 3.0, was incubated 5 minutes in a boiling water bath. Such a procedure would not be expected to appreciably affect RNase itself(10). The diluted serum was then cooled quickly, readjusted to neutrality, and assayed for RNase activity. There was no change in activity compared to an untreated guinea pig serum control. A sample of phosphate-buffered crystalline RNase at the same activity level as in the guinea pig serum was subjected to the acid and heat treatments described and lost more than half of its activity. The greater stability of the serum RNase is probably due to protection offered by the other proteins present.

Discussion. It is difficult to apply any sort of kinetics to this analysis of RNase activity because concentration of the infectious RNA substrate is extremely low. Since each plaque-forming unit probably represents one molecule of RNA(7), there are less than 5,000 molecules of RNA per ml. Increasing this amount by a factor of a thousand to allow for non-infectious RNA being present still yields a substrate concentration 1,000 times lower than the 4 x 10^9 molecules of RNase present in a 1:10,000 dilution of a 1 μg/ml solution. Thus, there is little possibility of the reaction being zero or first order as are the usual enzyme assay reactions. Instead, this assay should be treated as a chemical assay in so far as a standard curve using known concentrations of crystalline RNase is prepared for each series of determinations on unknowns.

It has been reported that in a 0.01% salt-free, tobacco mosaic virus RNA solution, addition of RNase (molar ratio, 15%) will pro-

TABLE III. RNase Content of Several Sera.

Serum	RNase content (μg/ml)	Serum	RNase content (μg/ml)
Human	.08-.10	Mouse	.08- .10
Bovine	.08-.10	Guinea pig	2.2 -3.0
Sheep	.08-.10	Rabbit	.05- .08
Swine	.10-.12	Chicken	.06- .08
Horse	.02-.05		

duce an inactive RNA complex which can be treated with phenol to regenerate an average of 20% of the original infectivity. Kirby (13) had shown previously that phenol will inactivate RNase. The recovery of infectivity decreased both when the ratio of RNase was changed and when salts were present (12). Comparable conditions do not exist in the enzyme-substrate system used in the present report since close to physiological conditions are employed with at least a 1000-fold higher concentration of enzyme. As shown by the failure to obtain infectious RNA from RNase treated FMDV-RNA, the RNase is hydrolyzing its substrate.

This assay for RNase might also be extended to make use of any infectious RNA for which infectivity can be accurately measured. A comparable assay for DNase could also be set up by using an infectious DNA such as the one recently obtained from polyoma virus(14).

The greatest advantage of this assay is in detection of trace amounts of RNase activity not measurable by any other method. Currently available methods of assay(1-6) are most convenient to use for microgram levels of enzyme. Only under favorable conditions with extended incubation times do these methods yield greater sensitivity. The biological assay reported here using infectious RNA invariably gave a sensitivity of 0.1 mμg of RNase/ml. In the analysis of unknowns, possible interfering RNA-inactivating materials can be removed by dialysis or acid and heat treatment, since RNase itself is resistant to these treatments. As shown in the present experiments, RNase activity in the sera of several animal species and of man was not due to any acid- or heat-labile or low-molecular-weight material. Comparison of the values obtained here with those reported in the literature for rat and rabbit sera(3,11) was not feasible because the latter values are reported in terms of cu mm of carbon dioxide.

Summary. An ultra-sensitive method of assay for ribonuclease (RNase) has been devised using a biologically active ribonucleic acid (RNA) as its substrate. Infectious RNA isolated from foot-and-mouth disease virus was exposed to suitable dilutions of RNase, and the resulting loss in RNA infectivity was measured by plaque assay in calf-kidney cultures. Concentrations of enzyme as low as 0.1 mμg per ml were readily detected. The RNase level in sera from man and several animal species was determined by this method. Most sera contained 0.02 to 0.1 μg/ml of RNase, but guinea pig serum had a 20- to 30-fold higher content of this enzyme.

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Relation of Mammotropes to Mammary Tumors. IV. Development of Highly Hormone Dependent Mammary Tumors.* (26152)

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There are two simple methods to produce mammary carcinomas in rats: administration of a chemical carcinogen (as 3-methylcholanthrene(1)) and ionizing radiation(2). The former, reported by Huggins(1), calls for repeated oral administration of the carcinogen. We found(3) that a subthreshold dose of this carcinogen produces mammary carcinomas in most rats whose mammary glands had been stimulated by mammotrope. Mammotropic hormone(s) alone did not produce mammary carcinomas. On the basis of this and other experiments (*cf*. 4) the theory was advanced that the hormone is a mere promoter or activator of neoplastic cells induced by a carcinogen, and that the altered cells can remain latent for long periods. Mammotropic hormone(s) not only revived the regressed or arrested mammary carcinomas following hypophysectomy but also brought about many new mammary carcinomas in the same hypophysectomized host(5).

In earlier studies nearly 80% of the mammary carcinomas induced by repeated administrations of 3-methylcholanthrene were found to be hormone dependent in the original passage(5) but upon successive transplantations rapidly gave rise to less and less hormone responsive autonomous variants. It is probable that when a tissue or organ is subjected to an intensive carcinogenic treatment numerous cells undergo malignant transformation, and, upon transplantations, the host selects the more malignant (autonomous) variants. We postulated that by reducing the amount of carcinogen and increasing that of the stimulating hormones, it may be possible to develop a much needed highly hormone dependent and relatively stable tumor strain.

Materials and methods. The 6 mammary carcinomas studied originated in the mammary tumor induction experiment by a combined treatment of a subcarcinogenic dose of 3-methylcholanthrene (10 mg) and a grafted functional mammotropic tumor (3, *cf*. Table III). Tumor transplantations were made by injecting 0.1 ml of tumor mince subcutaneously into highly inbred Wistar/Furth (W/Fu) rats, in which the tumors were induced. Mammary carcinoma mince was injected into the right inguinal mammary gland region and mammotropic tumor (Strain W2,(6)) in the back of the neck. The recipients were approximately 2 to 3 months of age. For estrogen treatment a pellet containing 0.5 mg diethylstilbestrol (DES) fused with 9.5 mg cholesterol was implanted subcutaneously into the back.

Results and comments. Five induced mammary carcinomas (W-5, 7, 9, 12, 16) were transplanted into W/Fu rats of both sexes. When 4 to 9 months after transplantation it was found that these tumors would not grow in either normal female or male rats, the rats were divided into 3 to 5 groups and were treated with DES or mammotropic tumor (to administer mammotropic hormone(s)), one group remaining untreated (control) as indicated in Table I. Treatment was initiated from 0 to 262 days after the mammary tumor transplantation.

With mammary carcinoma strain W-9, 3 successive sub-passages were made. All showed high mammotropic hormone dependency. None of the 5 strains could be transplanted to normal male rats and only 2 of 50 grafts (4%) were successful in untreated females. Transplantation was successful in 3 of 31 females (9.6%) treated with a DES pellet, while 50 of 56 females (89%) and all of 7 males that received mammotropic tumor developed a tumor.

In one of 6 carcinoma-bearing rats in which mammotropic tumor was completely resected, the mammary carcinoma promptly regressed

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TABLE I. Mammotropic Hormone Dependency of Mammary Carcinoma Induced by a Subthreshold Dose of 3-Methylcholanthrene and Mammotropic Hormone(s).

			♀			♂	
Mammary carcinoma strain	Passage	Days, graft to treatment	Normal	+ DES†	+ Mammotropic tumor	Normal	+ Mammotropic tumor
W-5	I	262	1/6*(321)	1/6 (321)	5/6 (322)	0/4	4/4 (330)
W-7	I	220	0/5	0/6	4/5 (278)		
W-9	I	143	0/7	1/6 (235)	5/6 (199)	0/5	
	IIa	17	0/10		14/14 (61)	0/4	
	IIIa	0	0/6		6/6 (36)	0/6	
	IIb	0	0/3		6/6 (120)		3/3 (122)
W-12	I	113	1/6 (197)	1/6 (197)	5/6 (181)		
W-16	I	0	0/7	0/7	5/7 (71)	0/2	
Total			2/50 = (4.0%)	3/31 = (9.6%)	50/56 = (89%)	0/21	7/7 = (100%)

* No. of takes over No. of rats inj. Numbers in parentheses indicate mean latent periods, in days.

† DES, 0.5 mg, in pellet subcut.

and never recurred, thus manifesting complete mammotropic hormone dependency.

Earlier experiments indicated that estrogen facilitates mammary carcinoma growth by way of stimulating mammotrope(6). The difference between DES and mammotropic tumor treatment is best explained by differences in mammotropic hormone level of the treated animals.

The latency of mammary carcinomas varied with time of mammotropic tumor graft. A comparison of the passages of the mammary carcinoma strains indicates a direct relation between time of mammotropic tumor graft and mammary carcinoma latency. Delay in mammotropic tumor graft prolonged appearance of grafted mammary carcinoma. When mammary carcinoma strain W-9 graft was followed immediately by mammotropic tumor graft, mammary carcinoma appeared in 36 days. But when mammotropic tumor graft was delayed by 17 and 143 days, mammary carcinoma did not appear until after 61 and 199 days, respectively. Tumor cells can remain latent for as long as 262 days and be brought to rapid proliferation by mammotropic hormone(s) (Table I).

Pituitary tropic hormones do not destroy cells but merely regulate their numbers. Correspondingly, hormone dependent tumor cells are not destroyed in histocompatible hosts. They can remain dormant in glands made hypoplastic by inhibitory hormones or by deprivation of stimulating hormones.

Dependent tumors are usually slow and autonomous tumors fast growing. Both latency and rate of growth depend on degree of hormonal stimulation.

The microscopic appearance of a highly hormone dependent tumor of this series showed slight morphologic "dedifferentiation." The tumors were well differentiated adenocarcinomas. The tumor cells and their nuclei were fairly uniform in size and shape, and there was but slight hyperchromatophilia and loss of polarity.

Summary. 1. Highly hormone dependent mammary tumors were induced in rats by a combined treatment of a subthreshold dose of 3-methylcholanthrene and mammotropic hormone(s) (administered by grafts of a functional mammotropic tumor). 2. Growth of these mammary tumors was dependent upon continuation of stimulation by mammotropic hormone(s). 3. Grafts of the mammary tumors remained latent up to 262 days, growing only after mammotropic hormone(s) was administered. 4. Upon resection of mammotropic tumor, a primary mammary tumor promptly and completely regressed; 5 other primary tumors in rats carrying functional mammotropic tumors grew progressively.

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Starch Gel Electrophoresis of Lactic Dehydrogenase from Rat Kidney.* (26153)

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Markert and Moller(1) and Tsao(2) have reported methods for localization of dehydrogenase activity after starch gel electrophoresis. Markert and Moller employed neotetrazolium as the terminal electron acceptor in a medium containing substrate, cofactor, methylene blue, diaphorase and hydrazine while Tsao employed the same tetrazolium, omitted diaphorase and replaced hydrazine with cyanide. Use of neotetrazolium necessitated the application of the incubating medium in the form of an agar(1) or starch gel(2) overlay to obtain anaerobic conditions and also required long periods of incubation.

This report describes an improved method for demonstration of lactic dehydrogenase (LDH) after starch gel electrophoresis based on the use of the more sensitive tetrazolium, Nitro BT, and substitution of phenazine methosulfate (PMS) for exogenous diaphorase. In addition, the standard incubating medium was varied in an attempt to characterize more closely the LDH fractions obtained from homogenates of kidneys of adult rats. Also, a technic is described for demonstration of diphosphopyridine nucleotide (DPN) diaphorase after starch gel electrophoresis.

Methods. Whole rat kidneys, homogenized in an equal volume of water, were frozen and thawed several times and centrifuged to remove tissue debris. A portion of the supernatant from each kidney was placed on a filter paper strip, inserted into a starch column, and subjected to electrophoresis for 6 hours(3,4).

Following electrophoresis, each column was sliced into 5 strips. One strip was incubated in the standard medium containing sodium lactate, 0.1M; DPN, 0.3 mg/ml; PMS, 20 μg/ml; Nitro Bt, 0.5 mg/ml; potassium cyanide, 0.1M; and phosphate buffer, 0.25M (pH 7.4) at 37° for one hour. In initial determinations, exogenous diaphorase was employed in the standard medium but this was subsequently replaced by PMS. Of the remaining strips, one each was incubated in a modification of the standard medium in which either DPN, PMS, or both DPN and PMS were omitted. DPN diaphorase activity was revealed by incubating a strip in a medium containing reduced DPN, 1.0 mg/ml; Nitro BT, 0.5 mg/ml; and phosphate buffer, 0.5M (pH 7.4). Malic dehydrogenase activity was demonstrated by substituting sodium malate for sodium lactate in the standard medium.

Results. Five distinct bands exhibited LDH activity when the column was incubated in the standard substrate (Fig. 1). Four of the bands migrated toward the anode while one band was on the cathodal side of the origin. When the column was incubated in the medium from which DPN had been omitted, bands 1, 4, and 5 could not be demonstrated, while either band 2 or band 3 was enzymatically active. In the absence of

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† Predoctoral Fellow, Nat. Cancer Inst., U.S. P.H.S.

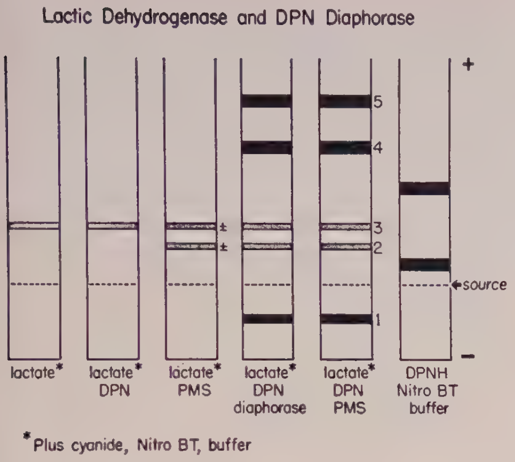


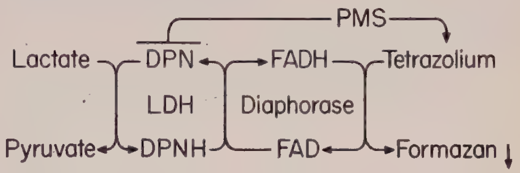
FIG. 1.

PMS or both DPN and PMS only band 3 could be demonstrated. Separation of bands 2 and 3 was often incomplete. The migratory rates of these bands is so nearly similar that a sufficiently long period of electrophoresis is required for their resolution. LDH fractions in homogenates of rat heart were similar to those of rat kidney except that all required DPN and PMS in the incubating medium.

DPN diaphorase activity, as demonstrated with exogenous DPNH, was not present in any of the LDH fractions but was revealed in 2 additional bands (Fig. 1). These occurred as a slow moving band between the origin and band 2 of LDH and a more rapidly moving band situated between bands 3 and 4 of LDH. The diaphorase bands also contained malic dehydrogenase.

Discussion. The present method offers distinct advantages over those previously employed(1,2). The use of Nitro BT affords a greatly reduced incubation time and obviates the necessity for anaerobiasis. Thus the present system does not require use of an agar or starch gel overlay. Cyanide was used as a carboxylic acid trap since hydrazine nonspecifically reduces Nitro BT under the conditions employed. However, cyanide was not essential for the reaction to proceed but did facilitate the reaction rate.

Use of phenazine methosulfate was suggested by the work of Singer and Kearney (5) and Farber and Bueding(6). Singer and Kearney(5) proposed that the site of action of PMS in transfer of electrons to an artificial acceptor is near or likely at the level of the dehydrogenase. The present work would indicate the site of action is the dehydrogenase since it was found that PMS and diaphorase were interchangeable as requirements for demonstration of certain of the LDH bands. The reaction sequence is assumed to be as follows:



The electrophoretic heterogeneity of tissue LDH has been clearly substantiated by the work of Wieland(7), Markert and Moller (1), Tsao(2), and the present report. The presence of 5 LDH fractions in rat kidney homogenates is in agreement with the findings of Markert and Moller(1) in adult porcine kidney with the exception of one band which migrated toward the cathode.

Certain of the LDH fractions of rat kidney homogenates were found to differ in cofactor requirement. Bands 1, 4, and 5 always required DPN and either exogenous diaphorase or PMS. Band 2 always required addition of diaphorase or PMS but variably required DPN. Band 3 did not need added diaphorase and also exhibited variation in its requirement for exogenous DPN. Since bands 2 and 3 varied inversely in their requirements for exogenous DPN this would suggest that endogenous DPN was present in either of the 2 bands, while endogenous diaphorase was present only in band 3.

Occurrence of bound DPN in various enzyme preparations has been reported by several workers(8,9,10). A less probable explanation would be the existence of a non-DPN linked enzyme. The failure to demonstrate endogenous diaphorase activity in LDH band 3 with reduced DPN as substrate, may be due to presence of a segment of the respiratory chain which is only capable of oxidizing DPNH generated *via* the dehydrogenase. The alternate possibility that this fraction does not function through a diaphorase would seem unlikely. Green(11) has demonstrated that the respiratory chain of beef heart mitochondria tends to fragment into several particles each of which contains several characteristic enzymes. In view of these findings it seems possible that certain of the LDH fractions, as demonstrated in starch gel, may consist of enzyme units. This is further suggested by the finding that the major diaphorase bands also contain malic dehydrogenase.

It is apparent that additional explanations for the electrophoretic heterogeneity of LDH must be considered before accepting the postulation that this heterogeneity represents different molecular species(1). Such possibilities as sulfhydryl or other protein interaction (12) or coupling of the dehydrogenase to various components of the respiratory chain cannot be excluded.

Summary. 1. An improved method for demonstration of lactic dehydrogenase after starch gel electrophoresis is described. This method employs a more sensitive tetrazolium and phenazine methosulfate. 2. Rat kidney contained 5 lactic dehydrogenase fractions which varied in cofactor requirements. DPN diaphorase was not demonstrable in any of these fractions, when reduced DPN was employed as the substrate, but was demonstrated in 2 additional bands. 3. The possibility is discussed that at least certain of the lactic dehydrogenase fractions from rat kidney exhibit electrophoretic heterogenity because of their association with various components of the respiratory system.

We are deeply indebted to Mrs. Julia Ternak for technical assistance.

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Neuromuscular Blocking Properties of Various Polypeptide Antibiotics.[†]
(26154)

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The neuromuscular blocking action of several antibiotics has been reported. Pittinger and associates(1,2) demonstrated the neuromuscular blocking action of neomycin sulfate and the enhancement of this effect by ether anesthesia. Brazil and Corrado(3) reported the "curariform" action of streptomycin. Timmerman and co-workers(4) reported neuromuscular blockade following administration of dihydrostreptomycin sulfate, polymyxin B sulfate and kanamycin sulfate. This report describes the neuromuscular blocking action of several polypeptide antibiotics and the effect of neostigmine methylsulfate and calcium chloride on it. Various constituents of the polypeptides were also evaluated for neuromuscular blocking activity.

[†] This work supported in part by a grant from the U.S.P.H.S. (RG-B-1396).

TABLE I. Chemical Composition and Neuromuscular Blocking (N.M.) Activity.

Antibiotic	Chemical composition*	ED$_{50}$, mg/kg (95% C.I.)	N.M. potency relative to colistin (95% C.I.)
Colistin	+ 6-methyloctanoic acid, D-leucine, L-threonine, L-leucine, L-α-γ diaminobutyric acid	9.1 (9.0- 9.2)	1.00 (——)
Polymyxin A	+ 6-methyloctanoic acid, D-leucine, L-threonine, L-α-γ diaminobutyric acid	14.0 (13.0-14.2)	.66†(.58- .70)
" B	+ 6-methyloctanoic acid, D-leucine, L-threonine, D-phenylalanine, L-α-γ diaminobutyric acid	5.7 (5.3- 6.0)	1.59 (1.53-1.64)
Neomycin	Diaminohexose moietie linked to D-ribose linked to neamine	34.2 (29.3-35.6)	.28 (.23- .32)
Viomycin	Creatinine, guanidino component, L-serine, α-β diaminopropionic acid	77.5 (77.1-78.0)	.12†(.09- .16)

* See ref. 7 and 9. † X^2 test for homogeneity of variance was non-significant at $p = .1\%$.

Methods. The following antibiotics were investigated: bacitracin, colistin sulfate, neomycin sulfate, polymyxin A sulfate, polymyxin B sulfate, ristocetin, tyrothricin, vancomycin hydrochloride, viomycin sulfate and sodium colistin methanesulfonate.* Solutions were freshly prepared and administered intravenously.

Neuromuscular blocking activity was evaluated using the sciatic nerve-gastrocnemius muscle preparation in the rabbit and chicken. Rabbits weighing 1.0-1.5 kg were anesthetized with sodium phenobarbital (200 mg/kg) and bipolar silver electrodes were placed on the peripheral end of the sectioned sciatic nerve. The nerve was stimulated for 0.2 sec with monophasic square wave pulses of 2 milliseconds duration at a frequency of 250 per sec and of supramaximal voltage delivered every 5 sec. A Grass stimulator (Model S4C) with circuit interrupter was used in all experiments. Semi-isometric recordings were made on smoked kymograph paper. Twelve to 19 rabbits were used to evaluate relative potency of each antibiotic. The dose producing 50% neuromuscular blockade (ED$_{50}$), potency ratios and their 95% confidence intervals were calculated as outlined by Finney (5) for unsymmetrical bioassays.

The effect of neostigmine methylsulfate (50 μg/kg) and calcium chloride (60 mg/kg) upon the neuromuscular blockade produced in rabbits was investigated. Antibiotics that produced blockade which was enhanced by neostigmine methylsulfate were studied in the chicken using the method described by Pelikan *et al.*(6). The parameters described for the rabbit sciatic nerve-gastrocnemius muscle preparation were also used in this preparation.

Various constituents of the polypeptide antibiotics producing neuromuscular blockade were also investigated in the rabbit sciatic nerve-gastrocnemius muscle preparation.

Results and discussion. Bacitracin, ristocetin, tyrothricin, vancomycin hydrochloride and sodium colistin methanesulfonate were administered to at least 2 rabbits at a dose of 100 mg/kg (except for bacitracin and tyrothricin, of which 25,000 units and 0.5 mg/kg, respectively, were given) and no neuromuscular blockade was observed. Colistin sulfate, neomycin sulfate, polymyxin A sulfate, polymyxin B sulfate and viomycin sulfate produced neuromuscular blockade as described below. The constituents of antibiotics studied, ED$_{50}$ and relative potency of the active antibiotics are shown in Table I.

Colistin sulfate. This antibiotic recently introduced in Japan as Colimycin is structurally related to the polymyxins. For purposes of assessing relative potencies, colistin sulfate was chosen as the standard and therefore assigned a value of 1.00. The ED$_{50}$ was 9.1 mg/kg. Neostigmine methylsulfate did not appear to be an effective antagonist of the neuromuscular blockade produced by colistin sulfate. Likewise, calcium chloride did not antagonize the blockade. Colistin sulfate neither produced contracture when evaluated in the chicken nor was the blockade antagonized by 50 μg/kg of neostigmine methylsulfate. The methylated form of colistin (sodium colistin methanesulfonate) is devoid of neuromuscular blocking activity. This compound has been reported to be rapidly eliminated by the kidneys(7), which may explain in part its lack of activity.

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* Colistin sulfate and sodium colistin methanesulfonate were kindly supplied by Warner Lambert Labs. Polymyxin A and + 6-methyloctanoic acid were obtained through the courtesy of Wellcome Research Labs.

Neomycin sulfate. The relative potency for neomycin sulfate was found to be 0.28. ED$_{50}$ was 34.2 mg/kg. It has been reported (1,2) that calcium chloride as well as neostigmine methylsulfate is an effective antagonist of the neuromuscular blockade produced by neomycin sulfate. Our findings confirm these reports. Furthermore, successful clinical application of the antagonists has been reported (8).

Polymyxin A Sulfate. Polymyxin A differs chemically from polymyxin B in that it lacks D-phenylalanine (Table I). Relative potency for this compound was found to be 0.66. Its ED$_{50}$ was 14 mg/kg. Neither calcium nor neostigmine methylsulfate were effective antagonists of the neuromuscular blockade produced by polymyxin A. Polymyxin A produced, in the chicken, a flaccid paralysis which was not antagonized by neostigmine methylsulfate.

Polymyxin B sulfate. This antibiotic was the most potent of the polypeptides studied. Relative potency and ED$_{50}$ were found to be 1.59 and 5.7 mg/kg, respectively. Neither neostigmine methylsulfate nor calcium chloride were effective antagonists. As with polymyxin A, a flaccid paralysis was produced in the chicken which was not antagonized by neostigmine methylsulfate.

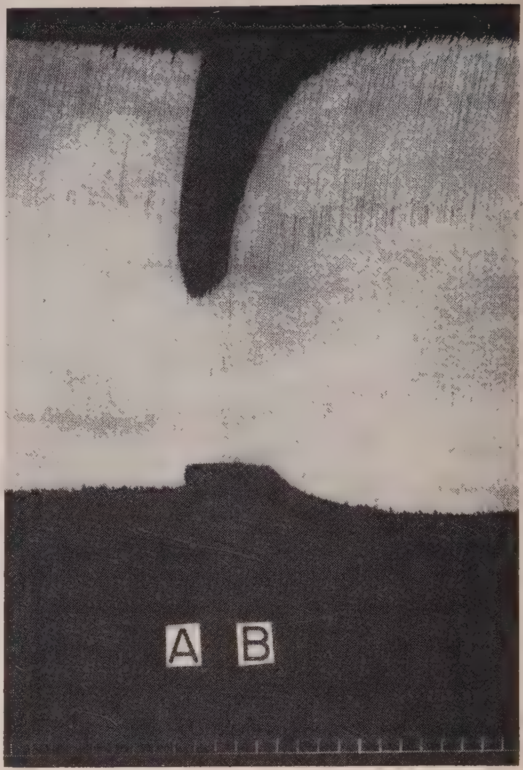

FIG. 1. Effect of neostigmine methylsulfate upon neuromuscular blockade produced by viomycin sulfate. Time marks equal 1 min. A. Viomycin sulfate, 90 mg/kg. B. Neostigmine methylsulfate, 50 μg/kg.

Viomycin sulfate. Of the compounds studied, viomycin sulfate appeared to be the least active. Relative potency and ED$_{50}$ were found to be 0.12 and 77.5 mg/kg, respectively. The antagonistic effect of neostigmine methylsulfate upon the neuromuscular blockade produced by viomycin sulfate is shown in Fig. 1. Calcium chloride was also found to be an effective antagonist of the blockade produced.

The various amino acids and other constituents comprising the antibiotics (Table I) were also investigated in the rabbit for neuromuscular blocking activity. The following compounds failed to produce neuromuscular blockade when evaluated using doses up to 100 mg/kg: D-leucine, L-threonine, L-leucine, α-γ diaminobutyric acid, D-phenylalanine, creatinine and L-serine. +6-Methyloctanoic acid when evaluated in doses up to 10 mg/kg showed no effect.

The preceding data demonstrate the neuromuscular blocking properties of several polypeptide antibiotics. The neuromuscular blockade produced by these agents was reversible and duration of action was dose related. Neuromuscular blockades of 85-95% returned to control levels in 20-40 minutes. The results obtained with various antagonists suggest that the polypeptides studied do not have the same mechanism of action. Neostigmine methylsulfate or calcium chloride were not effective antagonists of the neuromuscular blockade produced by polymyxin A sulfate, polymyxin B sulfate and colistin sulfate. However, these compounds were effective antagonists of the blockade produced by neomycin sulfate or viomycin sulfate.

The failure of the polypeptide components to produce neuromuscular blockade suggests that the intact peptide molecule is necessary for activity. If there is a critical distance between 2 nitrogen atoms necessary for neuromuscular blockade, as suggested by various investigators(10,11,12) then one might expect the necessity of the intact peptide molecule or at least more than a single amino acid to be necessary for neuromuscular blockade.

We are unaware of any clinical reports of respiratory depression resulting from the neuromuscular blocking action of these agents other than neomycin sulfate. However, it is conceivable that the other antibiotics could produce undesirable clinical effects under circumstances of overdosage, excessive absorption, or in presence of anesthetic agents capable of enhancing their neuromuscular blocking action.

Summary. Colistin sulfate, neomycin sulfate, polymyxin A sulfate, polymyxin B sulfate, and viomycin sulfate produced neuromuscular blockade when tested on sciatic nerve-gastrocnemius muscle preparations of the rabbit. Data obtained from the neuromuscular preparations were statistically analyzed and the relative potencies of the antibiotics in relation to colistin sulfate were determined. Polymyxin B sulfate was the most active antibiotic, approximately 1.5 times as active as colistin sulfate. Neostigmine methylsulfate antagonized the neuromuscular blockade produced by neomycin sulfate and viomycin sulfate, but was not an effective antagonist of the blockade produced by colistin sulfate, polymyxin A sulfate, and polymyxin B sulfate. Colistin sulfate, polymyxin A sulfate and polymyxin B sulfate do not produce contracture when administered to the chicken. The amino acids and other constituents comprising the various peptide antibiotics failed to show neuromuscular blocking activity.

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Abalone Juice. Fractionation and Antibacterial Spectrum. (26155)

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The antibacterial activity of crude fresh abalone juice has been reported(1,2). The active substance (or substances) was thermostable, non-dialyzable and not easily diffusible, suggesting a macromolecule. In view of the widespread use of cellulose ion-exchange chromatography for separation and purification of proteins from animal or other materials(3,4), it seemed advantageous to use this procedure for isolation of the antimicrobial material present in abalone juice. This report describes the isolation of several fractions and results of tests on the *in vitro* antibacterial action of these against *Staphylococcus aureus* and other pathogenic bacteria.

Materials and methods have been previously described(1,2) except the following additions: *Test organisms.* Four additional strains of bacteria, *i.e., Salmonella paratyphi, A and B, Salmonella typhi* and *beta-hemolytic Streptococcus pyogenes*§ were used as test organisms. *Fractionation procedure.* One hundred ml of crude abalone juice were exhaustively dialyzed against distilled water at 4°C for 5 days. The small amount of insoluble material accumulating was removed by centrifugation and discarded. The dialyzed extract was adjusted to pH 7.8 and applied to a column packed with 50 g of the anion-exchanger diethylamino-ethylcellulose (Cellex-D)‖ equilibrated with 0.01 M tris (hydroxymethyl) aminomethane-phosphoric acid (tris-H_3PO_4) buffer at pH 7.6. The column was set up on an automatic fraction collector in the cold (4°C). Elution of material from the column was carried out with a series of Tris-H_3PO_4 buffers of varying ionic strength and pH (Table I). Fifteen ml samples of effluent were consecutively collected in each of 200 tubes at a rate of 3 ml per minute (Table I). The column was then washed with one liter of M NaCl to elute the residual highly bound protein. Several pools were made of various samples, dialyzed against distilled water for 48 hrs in the cold and lyophilized. The final lyophilized products were dissolved in pH 7.0 buffered saline, passed through a very fine glass fritted filter or heated in a water bath at 70°C for 30 minutes before assay.

Results. Activity of various fractions. The data in Table I demonstrate that several fractions isolated from abalone juice by ion-exchange chromatography contained marked antibacterial activity. The greatest activity was found in the first 4 pools of early eluates, corresponding to tubes 11-50. Eluate from tubes 1-10 (150 ml) after dialysis and lyophilization revealed a negligible amount of material not enough to test and it was discarded. The fifth pool showed some activity while all later eluates including the final M-NaCl wash were inactive. Two additional chromatographic experiments were performed with juice from 1-3 fresh frozen abalones. Similar results were obtained, although there was some variation in quantity of yield and intensity of activity of the product from different abalones. For example, from 100 ml of crude abalone juice used in the first chromatographic separation, 52 mg of active product was obtained from the first 4 pools, whereas, in the second experiment the yield was 200 mg of a product with less than ¼ the activity. Whether this was due to variation in technic or variation in the contents of the abalone juice awaits further study.

Relationship between concentration and antibacterial activity. A series of 10-fold dilutions in buffered saline was prepared from

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* National Inst. of Allergy and Infect. Dis.

† Division of Biologics Standards.

§ These cultures were kindly supplied by Dr. E. B. Seligmann, Division of Biologics Standards and Dr. P. Geisler, NIH Clinical Path. Lab., respectively.

‖ Purchased from California Biochemical Corp., Los Angeles.

TABLE I. Antibacterial Activity of Various Fractions from Abalone Juice.

		Eluent Tris-H_3PO_4 buffer			Colonial growth of *Staphylococcus aureus* on plate†						
Fraction No.	Pool from tubes	Conc., molar	pH	Yield, mg*	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Control§
1	11- 19	.01	7.6	10	0	0	0	0	0	0	0
2	20- 30	.01	7.6	25	0	0	0	0	0	0	0
3	31- 40	.01	7.6	12	0	0	0	0	0	0	0
4	41- 50	.10	7.5	5	0	0	0	0	0	0	0
5	51- 89	.10	7.5	155	3	1	1	0	0	0	0
6	90-109	.25	3.7	115	3	3	2	0	0	0	0
7	110-149	.25	5.8	232	4	‖	3	0	0	0	0
8	150-179	.25	3.7	160	4	4	4	0	0	0	0
9	180-200	.25	3.7	125	4	4	2	0	0	0	0
10	M NaCl wash			4300	4	4	4	‖	4	0	0
	Control‡				4	4	4	4	0	0	0

1-4, etc. indicate degree of bacterial growth, 4 being heaviest growth. 0 = no bacterial growth.

* Lyophilized material.

† A series of 10-fold dilutions of *Staphylococcus aureus* culture (FDA 209 strain) was made and a loopful of each dilution was inoculated into a tube containing 1.8 ml of broth with 0.2 ml of abalone fraction. After 18 hr incubation a loopful from each tube was plated(2).

‡ Contained 0.2 ml of broth only.

§ Not inoculated.

‖ Tube broken.

a 100 mg % solution of fraction No. 2 (Table I), then tested *in vitro* against *Staphylococcus aureus* (Table II). It should be noted that concentration of the agent as low as 1 mg % showed activity. The same type of experiment was repeated with 2-fold dilutions of a 16 mg % solution of fraction No. 1 from the third chromatographic experiment with similar results.

Bactericidal activity of the product. Staphylococcus aureus inhibited for 48 hours in a broth culture by the presence of a fraction of abalone juice did not grow out when the culture was diluted with nutrient broth, 100 to 200 times and further incubated for a week. At this dilution, concentration of abalone fraction was far below that required for inhibition. It thus seems probable that the action of the agent under such *in vitro* conditions was bactericidal rather than bacteriostatic.

TABLE II. Antibacterial Activity of Pool 2 at Different Concentrations.

Final conc., mg %	Colonial growth of *Staphylococcus aureus* on plate* 10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	C†
100	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
1	4	4	4	1	0	0	0	0
.1	4	4	4	4	4	4	0	0
.01	4	4	4	4	4	1	0	0
Control	4	4	4	4	4	1	0	0

1-4, etc. indicate degree of bacterial growth.
0 = no bacterial growth.

* See Table I. † Control—not inoculated.

Antibacterial spectrum. The data presented in Table III indicate that fraction 2 from abalone was not only active against a penicillin sensitive strain of *Staphylococcus aureus,* but also effective against other Gram-positive and Gram-negative pathogens including a penicillin-resistant strain of *Staphylococcus aureus.*

Discussion. The antimicrobial agent reported here differed from the usual antibiotics usually obtained from fluid cultures of molds or allied microorganisms. It was not dialyzable, yet thermostable as reported previously (2) and probably protein in nature as it gave a positive biuret test and was precipitated by trichloracetic acid and other protein precipitants. Possibly we are dealing with a new class of agent which might be widely distributed in nature, particularly in sea animals and can be isolated for therapeutic and prophylactic purposes.

With these initial studies as background, further work on purification and chemical

TABLE III. Antibacterial Spectrum of Pool 2 from Abalone Juice.

Test organism	Conc., mg %	Abalone juice pool 2	Colonial growth of test organisms on plate* 10⁻¹	10⁻²	10⁻³	10⁻⁴	10⁻⁵	10⁻⁶	Control†
Staph. aureus, Pen. sensitive	5	+	0	0	0	0	0	0	0
		—	4	4	4	4	4	0	0
Staph. aureus, Pen. resistant	5	+	1	0	0	0	0	0	0
		—	4	4	4	4	4	0	0
Staph. aureus, Pen. resistant	10	+	0	0	0	0	0	0	
		—	4	4	4	4	4	0	0
Strep. pyogenes, beta hemolytic	5	+	2	0	0	0	0	0	0
		—	4	4	4	4	4	0	0
Salmonella paratyphi A	10	+	0	0	0	0	0	0	0
		—	4	4	4	4	4	4	0
Salmonella paratyphi B	10	+	1	0	0	0	0	0	0
		—	4	4	4	4	4	0	0
Salmonella typhi	10	+	1	1	0	0	0	0	0
		—	4	4	4	4	4	4	0

1-4, etc. indicate degree of bacterial growth. 0 = no bacterial growth.
* See Table I. † Not inoculated.

structure of the active agent and *in vivo* studies are in progress.

Summary. Ten fractions have been isolated from abalone juice by ion exchange chromatography (Cellex-D) and tested for *in vitro* antibacterial activity against *Staphylococcus aureus.* Four fractions of effluent showed high activity. One active fraction was tested and found effective against *Staphylococcus aureus,* (including a penicillin-resistant strain), *Streptococcus pyogenes,* beta-hemolytic strain, and *Salmonella typhi,* and *paratyphi A and B.* The action for *Staphylococcus aureus* seemingly was bactericidal.

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Increased Nutritional Requirements of *Saccharomyces cerevisiae* as a Result of Incubation at 38°C.* (26156)

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During nutritional studies on yeasts, it was noted that certain strains grew poorly or not at all at 38°C in a chemically defined medium which supported excellent growth at 30°C. In contrast, some of the strains grew equally well at 30°C or 38°C when inoculated into a complex medium. In most instances where such a phenomenon had been demonstrated previously, an increase in temperature was accompanied by an increase in nutritional requirement(1-7). The present paper is concerned with identification of the substance(s) in the complex medium which

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TABLE I. Effect of Temperature of Incubation on Growth of Yeasts in a Complex Medium.*

Yeast strain	Turbidity (24 hr) 30°C	38°C
S. cerevisiae Java	86	67
" (Fleischmann strain 139)	110	78
" (ATCC† 2338)	84	72
S. globosus (" 10600)	99	3
S. carlsbergensis (" 9080)	91	47
S. fragilis (" 10022)	87	90
S. intermedius (" 2360)	77	2

* ACG medium consisting of 1.0% yeast extract, 1.0% casitone, 0.5% K_2HPO_4, and 0.5% glucose (pH 7.0).

† ATCC = American Type Culture Collection, Washington, D. C.

permitted growth of yeasts at the higher temperature.

Materials and methods. Several species and strains of *Saccharomyces* were used, but most of the studies were performed with *S. cerevisiae* (Fleischmann Strain 139). The yeasts were maintained in a complex medium (see Table I for composition); the experimental medium was a modification of the one introduced by Snell, *et al.*(8) and contained 10^2 μg/liter of biotin.

All yeasts were cultivated in the complex medium at 30°C and were transferred at approximately 24-hour intervals 3 or 4 times prior to use in an experiment. The cells were harvested by centrifugation and washed 3 times with 5.0 ml of sterile distilled water.

The experimental culture tubes were 22 x 150 mm screw-cap tubes with caps loosely fitted. Materials to be investigated were added aseptically along with 4.0 ml of medium and distilled water to bring the final volume to 5.0 ml. Inoculum was added in the amount of 0.05 ml. The criterion for growth was turbidity, as measured in a Klett-Summerson photoelectric colorimeter (660 mμ) using the medium as a blank. Incubation for about 24 hours was carried out in water baths where temperatures were maintained to within ± 0.5°C.

Results. Cultivating the organisms listed in Table I in Snell's medium at 38°C gave rise to somewhat surprising results, since only *S. fragilis* proliferated at this temperature. Since it was known that some of these strains were capable of growth at 38°C in a complex medium, it was necessary to compare their growth at 30°C and 38°C in a complex medium. The results (Table I) revealed that all but 2 of these strains were capable of growth at 38°C. These data suggested pH and/or nutrition as major factors related to growth of yeasts at 38°C. Further experiments in which Snell's medium and ACG medium at pH's of both 4.0 and 7.0 were tested for their capacity to support growth at the elevated temperature made clear that pH is not a factor in the growth response of these organisms at 38°C.

Before the effect of nutrition could be inspected, it was necessary to investigate the influence of inoculum size and to standardize the number of yeast cells to be used in subsequent experiments because 1) preliminary results employing bulk inoculations were somewhat erratic; 2) a rough titration of inoculum size suggested that initial number of cells present affected the expression of the temperature phenomenon; and, 3) past experience in this laboratory(9) as well as others(10) has shown that inoculum size can be of critical importance in nutritional work.

A suspension of yeast cells was prepared as previously described and a series of dilutions inoculated into several media and incubated at the 2 temperatures. Fig. 1 shows that inoculum size was an important factor in the phenomenon under investigation. For ex-

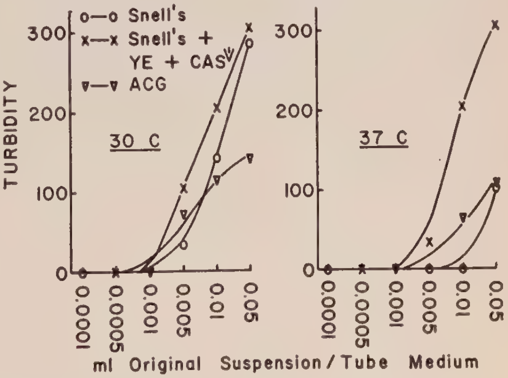

FIG. 1. Influence of inoculum size on growth of *Saccharomyces cerevisiae* (139) at 30°C and 38°C in several types of media.

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[1] YE = yeast extract, 1.0%; CAS = casitone, 1.0%. All media adjusted to pH 4.0. Incubation time, 24 hr. Inoculum was 0.05 ml of dilution desired.

ample, at 30°C with an inoculum size of 0.01 ml, growth was obtained in the 3 media used, whereas at 38°C only Snell's medium plus yeast extract and casitone supported good growth. Standardization of inoculum size for all further studies resulted in using 0.05 ml of a washed suspension of yeast cells having a turbidity of 30.

As to the nutritional aspect, it was necessary first to determine whether the growth promoting substance(s) was organic or inorganic in nature. To this end an ashed mixture of yeast extract and casitone was tested and found to have no effect on growth of the organism at 38°C.

Inasmuch as a mixture of yeast extract and casitone was effective in allowing proliferation of *S. cerevisiae*(139) at 38°C, it was important to determine which of the 2 complex materials possessed the active substance (s). Moreover, since yeast extract is often regarded as a source of vitamins and other growth factors, a mixture of known B-vitamins was included.

Good growth recovery at 38°C was obtained with addition to Snell's medium of a vitamin solution, a mixture of yeast extract and casitone, yeast extract alone, or high concentrations of casitone (Table II). The growth recovery obtained upon addition of yeast extract suggested that a vitamin might be the stimulatory substance. This was substantiated by the results using the mixture of known B vitamins. Examination of the spectrum of B vitamins present in the vitamin mixture revealed that pantothenate was the most active single organic material concerned with the ability of yeasts to proliferate at 38°C (Table III). Repeated experiments demonstrated that addition of this vitamin supported growth at 38°C to the extent of 40 to 82% of that obtained at 30°C.

TABLE II. Growth of *Saccharomyces cerevisiae* (139) at 30°C and 38°C in Media of Different Composition.

Additions to Snell's medium	Turbidity (24 hr) 30°C	38°C
None	252	75
Vitamin solution*	246	188
" " , diluted 1:10	262	206
Yeast extract + casitone, 50 mg each	271	256
Idem 5.0 " "	299	256
" 0.5 " "	266	105
Yeast extract, 50 mg	275	276
" " , 5.0 "	296	237
" " , 0.5 "	266	121
Casitone, 50 mg	315	242
" , 5.0 "	273	60
" , 0.5 "	254	44

* 1.0 ml of vitamin solution contains: 20 μg *p*-aminobenzoic acid, 20 μg pantothenate, 40 μg pyridoxine, 20 μg nicotinic acid, 20 μg riboflavin, 20 μg thiamine, 10 μg folic acid.

Since growth was determined as a function of the total mass of synthesized protoplasm (*i.e.*, turbidity), it was important to evaluate growth by measurement of number of viable yeast cells. For this purpose the conventional plating method was employed. Results showed an excellent correlation between turbidity and viable count measurements at 38°C. Furthermore, death of the yeast cells occurred at a more rapid rate at 38°C than at 30°C in media lacking pantothenate. Since such death did not commence until at least 36 hours of incubation, the use of turbidity as a measure of growth coupled with reading the tubes at about 24 hours appears both adequate and accurate.

The results presented thus far suggest that the synthesis of pantothenate normally carried out by *S. cerevisiae*(139) at 30°C is in some manner impaired at 38°C. As a consequence of this, and the results obtained by Maas and Davis(6), it was desirable to inspect the precursors in the accepted synthesis of pantothenate by microorganisms, namely β-alanine and pantoic acid.

Since β-alanine is an obligatory requirement for growth of *S. cerevisiae*(139) in absence of pantothenate, it was inspected only briefly. Its presence did not alter the temperature effect in the usual concentration and the possibility of a competitive inhibitor of β-alanine being synthesized at 38°C was ruled out, since increasing the concentration of this amino acid almost 200-fold had no effect on growth.

Experiments designed to explore the efficacy of pantoate in reversing the temperature phenomenon revealed that while pantoyl lactone had very little activity, sodium pantoate was as effective as pantothenic acid itself al-

TABLE III. Comparative Effectiveness of Pantothenate, Pantoyl Lactone, and Sodium Pantoate in Permitting Growth of *Saccharomyces cerevisiae* (139) at 38°C.

Additions to Snell's medium	Concentration of additions, μM/ml	Turbidity (24 hr)	
		30°C	38°C
None	—	222	32
Pantothenate	10^{-5}	223	35
”	10^{-4}	220	40
”	10^{-3}	224	105
”	10^{-2}	224	120
”	10^{-1}	222	140
”	10^{0}	223	145
”	10^{1}	230	139
”	10^{2}	234	143
Pantoyl lactone	10^{-1}	228	33
” ”	10^{0}	222	31
” ”	10^{1}	226	41
” ”	10^{2}	222	67
Sodium pantoate*†	10^{-2}	224	33
” ”	10^{-1}	226	54
” ”	10^{0}	226	118
” ”	10^{1}	220	123
” ”	10^{2}	230	143
Yeast extract	0.1 g/ml	305	274

* NaCl controls were negative.

† Prepared from commercially available pantoyl lactone after the method of Maas(5).

beit at a much higher concentration (Table III). Yeast extract was considerably more active than either pantoate or pantothenate. The poor activity of pantoyl lactone when compared to pantoic acid is consistent with the observations of Maas(11), that the latter has about 3 times the effectiveness of the former both in reversing salicylate inhibition and supporting growth of a pantoate auxotroph of *Escherichia coli.*

Studies of the Java and ATCC 2338 strains of *S. cerevisiae* revealed that the former responded in a manner similar to the 139 strain, whereas only yeast extract was effective in promoting growth of the 2338 strain at 38°C.

Discussion. The results of experiments designed to elucidate the nature of the observed temperature sensitivity revealed that the effect of complex materials such as yeast extract was largely replaceable by pantothenic acid. These findings led to an inspection of the synthesis of pantothenate in the hope that the biochemical lesion might be identified.

The accepted pathway of pantothenate biosynthesis is the coupling of β-alanine with pantoic acid:

$$\begin{tikzcd}
\beta\text{-alanine} + & \text{pantoic acid} \arrow[r, "A"] & \text{pantothenate} \\
& X \arrow[u, "B"'] &
\end{tikzcd}$$

β-Alanine was studied at concentrations up to 200 times greater than that normally used in the chemically defined medium with entirely negative results. Pantoic acid, on the other hand, was as effective as pantothenate in preventing the temperature effect, but the concentration required was 100 to 10,000 times as great. These findings, in light of the pathway cited above, suggest that since pantoic acid effectively overcomes the temperature effect, either enzyme A is not temperature sensitive or possibly another mechanism of pantothenic acid biosynthesis is operative in this organism. Lansford and Shive (12) considered the possibility that pantonine and β-alanine would couple to yield pantonyl-β-alanine with subsequent deamination of the pantonyl moiety to yield pantothenic acid. Possibly the enzymatic synthesis of pantonine from pantoic acid is temperature sensitive.

The large amount of pantoic acid required as compared to the quantity of pantothenic acid needed, suggests that a competitive type of enzyme inhibition might be involved in the temperature phenomenon. The site of such competition might be in one of the series of reactions leading to formation of pantoate (site B), or conceivably in an entirely unrelated biochemical reaction which is temperature sensitive, thus resulting in accumulation of a substance that exercises its effect either at the site of β-alanine-pantoate coupling (site A) or, again, in a reaction leading to synthesis of pantoate. The identity of such a presumed inhibitor has not been established.

Summary. Data were presented revealing that the paucity of growth of certain strains of yeast at 38°C is somehow related to a reduced ability to carry out the biosynthesis of pantothenic acid. Results were discussed in the light of the accepted pathway of pantothenate biosynthesis. Since the organisms are genetically endowed with the enzymic make up to synthesize pantothenate, this provides another example of the fact that environment plays an important role in genetic expression.

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A Method of Crystallization of Human Myoglobin.* (26157)

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Myoglobin was first crystallized by Theorell(1) using material of equine origin. Human myoglobin was first crystallized by Drabkin(2). Additional methods of crystallizing human myoglobin have been described by Theorell and de Duve(3) and by Rossi-Fanelli(4). In these methods hemoglobin and other proteins are salted out of aqueous extracts of muscle by lead acetate, phosphate, or ammonium sulfate. Concentration of phosphate or sulfate is then gradually increased by dialysis(3) against concentrated solutions of these salts or by evaporation(4) and crystallization is thereby induced. These steps are performed at a constant hydrogen ion concentration near pH 6.6, the isoelectric point of human myoglobin(5).

We have had difficulty in regularly obtaining crystalline human myoglobin by these methods. We have developed a simple method of isolation and crystallization of human myoglobin that depends on precipitation of contaminating proteins from aqueous extracts of muscle at pH 8 by saturation with ammonium sulfate. Myoglobin remains in solution under these conditions. After precipitation of these contaminating proteins and adjustment of the pH to 7, myoglobin crystallizes out of solution.

Procedure. Skeletal muscle from surgical or autopsy sources was frozen within one hour of operative removal or post mortem examination, and stored at –60°C. Cases with a post mortem interval before autopsy of more than 4 hours or with debilitation were avoided. While still frozen, samples weighing approximately 100 g were chopped into small pieces and mixed in an electric blender with 2 ml of distilled water per gram of tissue. These and all subsequent steps were performed at 4°C. After blending, the mixture was adjusted to pH 8 with 2N sodium hydroxide and extracted for one hour with constant stirring. Insoluble materials were then removed by centrifugation at 16,000 g for 30 minutes and the supernate filtered through coarse filter paper.

The filtrate was then made 80% saturated by addition of solid ammonium sulfate, C. P. grade. During this and subsequent additions of ammonium sulfate, the solution was maintained at pH 8 ± 0.05 by additions of 2N sodium hydroxide or 2% acetic acid. The material was then centrifuged at 16,000 g for 30 minutes.

The supernatant fluid was brought to

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100% saturation by addition of excess ammonium sulfate and allowed to stand overnight with constant stirring. The material was centrifuged at 39,000 g for 30 minutes, precipitate discarded and supernate again stirred for 4 to 8 hours with excess ammonium sulfate. Centrifugation was repeated for 30 minutes at 39,000 g and any precipitate discarded.

The supernate was adjusted to pH 7.0 with 2% acetic acid and allowed to stand in beakers at 4°C. Crystallization began after 24 to 48 hours or occasionally longer. The initial precipitates were sometimes amorphous and were discarded after separation by centrifugation. The resultant supernate was again allowed to stand in beakers at 4°C. The crystals were collected by centrifugation or filtration.

Results. The resultant crystals were needle-shaped and doubly refractile. Crystals were dissolved in distilled water or phosphate buffer, pH 7, 0.1 ionic strength. The solutions were reduced with sodium hydrosulfite and converted to carboxymyoglobin. Absorption maxima in the 540 mμ and 580 mμ regions were determined in a Beckman DU spectrophotometer and were compared to a carboxyhemoglobin standard. The peaks corresponded to those given in the literature.

Discussion. Morgan(6) in his studies of the solubility of equine myoglobin in concentrated ammonium sulfate solutions and in concentrated phosphate buffers noted that at pH 6.6 myoglobin is much more soluble than hemoglobin and that ammonium sulfate or phosphate solutions may be utilized for quantitative separation of these proteins at this pH. Previous methods(3,4) for purification of human myoglobin have utilized unsaturated phosphate or ammonium sulfate solutions to precipitate hemoglobin from myoglobin solutions at a constant pH of about 7. In Theorell's method(3) proteins other than hemoglobin and myoglobin were first precipitated by basic lead acetate. In these methods(2,3,4) myoglobin was crystallized after removal of hemoglobin by gradually increasing ammonium sulfate or phosphate concentration at constant pH. In Theorell's method(3) additional purification by electrophoresis was required before crystallization was accomplished.

The present method of purification and crystallization depends on our observation that human myoglobin is soluble in saturated ammonium sulfate at pH 8 and that hemoglobin is insoluble under these conditions. After removal of other proteins, including hemoglobin, and adjustment to pH 7, myoglobin crystallizes out on standing. In our hands this method has proved simple and effective.

Summary. A method of purifying and crystallizing human myoglobin is described. This method depends on the observation that myoglobin is soluble in saturated ammonium sulfate solutions at pH 8.

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Diethylstilbestrol-Induced Intrahepatic Bile Duct Hyperplasia in the Rabbit.* (26158)

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While investigating the effect of carbon tetrachloride-induced liver insufficiency and iatrogenic hyperestrogenism upon the renal glomerulus, previously unreported changes in the intrahepatic bile ducts were produced. This report deals with severe hyperplasia of the intrahepatic bile ducts of rabbits receiving diethylstilbestrol. Similar changes have not been found in over 600 rabbits from the same source previously examined histologically in this laboratory. In addition, neither spontaneous hyperplasia nor development of cholangioma in the rabbit has been recorded (1). This study, therefore, indicates a previously unreported effect of diethylstilbestrol on the liver.

Materials and methods. Young adult, male, albino rabbits of a mixed strain, weighing initially 1700-4000 g, were used. All rabbits were fed a standard rabbit pellet from which the usual vitamin-antibiotic supplement was omitted.† The rabbits were divided into 4 groups. The first group of 10 rabbits each received 1 ml/kg initial body weight, of a 1:1 mixture of carbon tetrachloride and olive oil (CCl_4) twice weekly, subcutaneously. The rabbits were treated for 100-200 days until cirrhosis, as indicated by abnormal liver function tests, was obtained. Cirrhotic rabbits were then given both CCl_4 and one mg of diethylstilbestrol‡ in 0.2 ml of corn oil (DES) subcutaneously each day for an additional 50-100 days. The second group of 9 rabbits were each given CCl_4 and DES concurrently for 50-200 days. The third group of 10 rabbits received only DES for 50-200 days. The fourth group of 6 rabbits served as controls. All rabbits were subjected to complete postmortem examination.

Results. All rabbits of Groups 1, 2, and 3, which received DES for 50 days or more, regardless of other treatment, showed a distinctly abnormal bile duct proliferation. In most animals the proliferation was consistent with a severe reactive hyperplasia, while in one animal which received only DES the proliferation was suggestive of neoplasia. The animals in Group 1 were all severely cirrhotic at time of autopsy. Portal areas were enlarged by fibrous tissue and lobules were transected by thick fibrous bands connecting the portal areas. Within the portal areas and the fibrous bands there was a moderate to marked proliferation of small but well-circumscribed bile ducts which often almost completely filled the portal areas. Although polarity of cells was maintained the ducts appeared nonfunctional and the lumina were frequently nonexistent. Animals in Group 2, which received both CCl_4 and DES concurrently, developed only a mild cirrhosis but showed mild to moderate bile duct proliferation. Animals in Group 3 which received only DES were free of cirrhosis but showed the most severe degree of bile duct proliferation. In most animals there was a moderate to severe hyperplasia of the bile ducts which not only filled the portal areas but extended into the lobules and often completely across the lobule connecting adjacent portal areas. One animal from this group showed a severe proliferation of the bile ducts suggestive of neoplasia. In this rabbit which received DES for 200 days the liver had a "cauliflower-like appearance" with nodules varying from 1-4 mm in diameter. Microscopically the lesion consisted of massive proliferation of bile ducts with invasion of lobules, replacing approximately 80% of the liver cells. The proliferating cells were occasionally formed into recognizable biliary ducts with an intact

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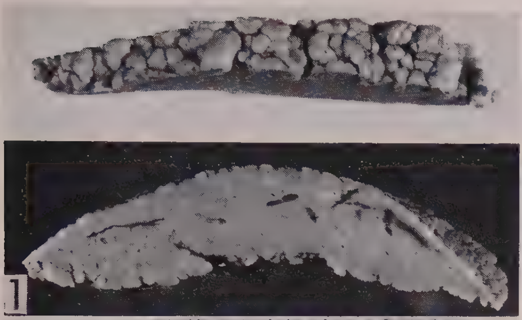
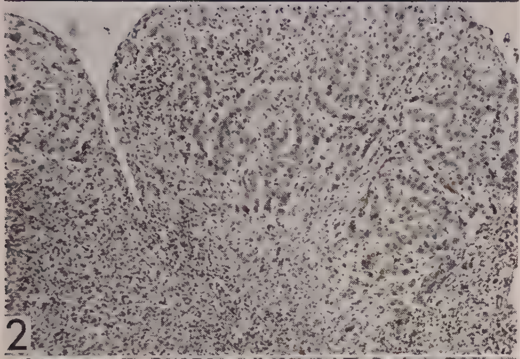
† Red Rose Rabbit Pellets—O.S.U. Formula, purchased from John W. Eshelman and Sons., Circleville, O.

‡ Donated by Dr. William R. Kirtley, Eli Lilly and Co.





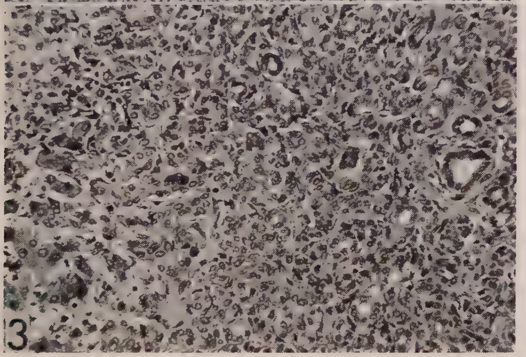


FIG. 1. Gross appearance of liver of rabbit from group 3.

FIG. 2. Microscopic appearance of liver of rabbit from group 3 which showed almost complete replacement of liver cells.

FIG. 3. Higher magnification of liver. A few remaining liver cells are present at left and a normal bile duct at right of photograph.

basement membrane, but more often consisted of irregular cords or sheets of cells in a connective tissue stroma. Polarity of cells was disrupted and in several areas blood vessel invasion was suspected but no distant mestatases were found. The control animals in Group 4 showed an occasional mild cholangitis but no evidence of cirrhosis or bile duct proliferation. All rabbits receiving CCl_4, Groups 1 and 2, lost weight while those rabbits receiving only DES, Group 3, and the control rabbits, Group 4, showed a normal growth curve. Subcutaneous abscesses developed at sites of injection of both CCl_4 and DES. Adrenal cortical hyperplasia was present in all rabbits receiving either CCl_4 or DES, Groups 1, 2, and 3. Testicular atrophy was present in all rabbits receiving DES, Groups 1, 2, and 3.

Discussion. Hyperestrogenemia is known to cause many growth abnormalities(2-10). However, the effect varies depending upon species of animal, estrogen level, dosage schedule, and type of estrogen administered. Hyperplasia, polyp formation and carcinoma of the endometrium, adenomyosis, uterine and extrauterine leiomyomas and fibromas, carcinoma of uterine cervix, adenoma and adenocarinoma of breast, hyperplasia of prostatic stroma, interstitial cell tumors of testis, chromophobe adenoma and adenoma of pars intermedia of the pituitary, lymphosarcoma of thymus, lymphomas, adrenal cortical neoplasms, and bone neoplasms have been reported after estrogen administration(2,8-10). Kirkman(11) reported that ethinylestradiol would produce hepatoma of the liver cells in hamsters. However, he was unable to produce similar changes with diethylstilbestrol and stilbestrol benzoate. Dow(4) found only fatty and hydropic degeneration of the livers of cats given estrogens for extended periods. No reports have been found of experimental estrogen-induced bile duct hyperplasia or neoplasia. The normal estrogens are inactivated and excreted in the bile(12). Synthetic estrogens such as diethylstilbestrol are more resistant to intrahepatic inactivation and are largely excreted intact, and reported, therefore, to be more tumorigenic(8). Some reports(7,13-16) state that inactivation of estrogens is decreased when the liver is partially excised or damaged by carbon tetrachloride while Cantarow *et al.*(17) disagree with this claim. Our data suggest that liver damage actually decreases the effect of estrogens on bile duct epithelium. Bile duct hyperplasia and an increased incidence of carcinoma of liver are often present in human patients with cirrhosis of the liver, most of whom also exhibit hyperestrogenemia. However, similar bile duct hyperplasia was not observed in cirrhotic rabbits receiving only CCl_4 in this laboratory. Estrogen has been reported to cause a decrease in growth, decrease in body weight of adult animals, increase in basal metabolic rate, hair loss, testicular atrophy, infertility, and hyperplasia of pituitary, thyroid and adrenal(3-7). In view of the well-established effect of estrogens on growth processes and excretion of estrogens in the bile, one might expect hyperestrogenemia to cause changes in bile duct epithelium. However, due to the body-wide endocrinopathy produced by estrogens one can only speculate as to whether the bile duct changes are due to a direct toxic effect of the estrogen, which seems most plausible, or to some other endocrine abnormality.

Summary. Administration of diethylstilbestrol to intact rabbits, or to rabbits made cirrhotic with carbon tetrachloride, produced in all animals a marked bile duct proliferation and in one animal changes suggestive of neoplasia of the bile ducts. The possibility that hyperestrogenism may be responsible for the increased incidence of carcinoma of the liver in cases of liver cirrhosis is suggested.

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An Improvement in Quantitative Separation of Phospholipids with Silicic Acid Impregnated Filter Paper. (26159)

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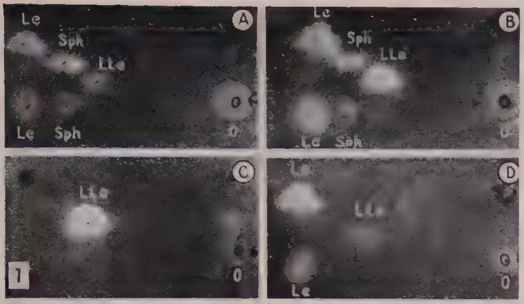
Recent developments in filter paper chromatography and the perfection of micro technics of analysis for specific substances have made it possible to quantitate compounds separated on paper. We have developed a simple method for quantitating minute amounts of the phospholipids, lecithin, lysolecithin, and sphingomyelin, following chromatographic separation on silicic acid impregnated filter paper, which uses the solvent systems and identification technics developed by Marinetti and coworkers(1,2). However we found that some of the lipid material from serum extracts, which was allowed to dry at point of application, became bound to the silicic acid at the origin and did not move with the chromatographic solvents employed. In addition to staining with Rhodamine G, it gave a positive test for choline. We avoided loss of this material, which was principally lecithin, by a technic we call "running start" chromatography. The technic is applicable to phospholipid analyses in tissue extracts as well as various biological fluids.



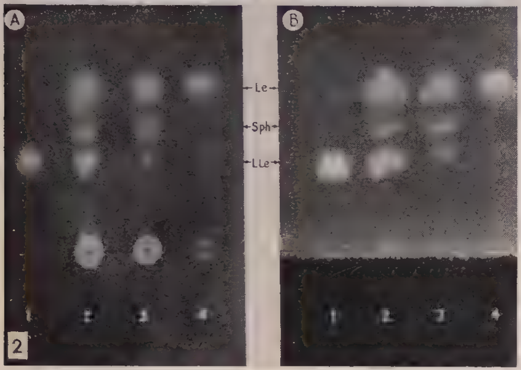


FIG. 1. Two dimensional separation of phospholipids on silicic acid paper. Applications of phospholipids in each of 4 chromatograms were: A, an extract of .02 ml serum containing 525 mg/100 ml phospholipids; B, same as A with 20 μg each of lecithin and lysolecithin added; C, 40 μg lysolecithin; D, 40 μg lecithin (relatively pure with slight contamination by lysolecithin). First separation (right to left) was with diisobutylketone : acetic acid : water 40 : 30 : 7 (v/v) for 11 hr at 20°C. After drying the paper, second separation (bottom to top) was with n-butyl ether : acetic acid : chloroform : water 40 : 35 : 6 : 5 (v/v) for 6 hr at 20°C. 0 = point of original application of samples. Direction of first solvent flow was right to left. Separated components after each solvent separation indicated by: Le, lecithin; LLe, lysolecithin; Sph, sphingomyelin. (Photographs of Rhodamine G stained chromatograms taken under ultraviolet illumination.)

FIG. 2. Contrast between "running start" and standard procedure for separating phospholipids on silicic acid impregnated paper, as exemplified with an extract of blood serum. Solvent used was diisobutylketone : acetic acid : water 40 : 30 : 7 (v/v). Chromatogram A (standard procedure) was run for 19½ hr, and B ("running start" procedure) for 16 hr at 11°C. Both stained with Rhodamine G and photographed under ultraviolet illumination. Point of application in B is approximately 2.0 cm lower than that in A. Applications at origins in both A and B were: 1, 40 μg of lysolecithin; 2, serum extract enriched with 20 μg each of lecithin and lysolecithin; 3, serum extract alone; 4, 40 μg of lecithin (relatively pure but with slight contamination by lysolecithin).

Methods. Whatman #1 filter paper was impregnated with silicic acid as originally described(1) in the cases of the chromatograms of Fig. 1 and 2, and as later modified (2) in the case of the data of Table I. An aliquot of an alcohol:ether 3:1 (v/v) extract of the material to be analyzed was evaporated and redissolved in isoamylalcohol:benzene 1:1 (v/v). A suitable proportion of this solution was applied to the origin. Diisobutylketone:acetic acid:water 40:30:7 (v/v) was our usual solvent. The chromatograms of Fig. 1 and 2 were developed in 6″ diameter jars and those for the data of Table I in a Kurtz-Miramon chromatographic apparatus, both not lined. Rhodamine G rather than 6G was used for staining the developed chromatograms. Elution of the phospholipid was accomplished with 1N HCl in methanol(2). The individual phospholipid spots were cut out and extracted in a 15 x 150 mm test tube with 3 successive volumes of 2 ml 1N HCl in methanol, bringing the solvent to the boiling point for 10-15 seconds. Each 2 ml extract was successively evaporated in a second 15 x 150 mm test tube placed in a steam heated water bath. The small residual volume was removed by placing the tube in a heating block at 130°C for 30 minutes. Lipid phosphorous was measured by a modification of the method of Beveridge and Johnson(3) in which volumes were reduced by one-tenth. One μg phosphorous yielded an absorbancy of 0.173 in 1 cm cells. Silicate from the paper has not interfered with the determination.

Results. In one-dimensional runs with pure

TABLE I. Recovery of Lecithin at Origin after Phospholipid Chromatography on Silicic Acid Paper.*

Lecithin applied, μg P	Recovery in separate runs, %	Lecithin applied, μg P	Recovery in separate runs, %
.24	21	1.20	19, 10, 12
.48	31	2.40	8
.60	12	3.60	6
.72	26	4.80	6
.96	25, 10, 14	6.00	8†

* Mixture of 3 phospholipids as described in the text were applied in a volume of 10 μl or by repeated additions to a total volume of 50 μl. Areas of application were essentially equal. Chromatograms were run for 16 hr at 17°C. After chromatography the amount of phosphorus remaining at the origin was measured and is reported as percentage of lecithin phosphorus applied.

† Overloaded origin spot. Value a little high.

lecithin, sphingomyelin, and lysolecithin, only lysolecithin moved completely from the point of application. In 2-dimensional runs with different solvent systems, lecithin and sphingomyelin left origin spots at both first and second separations (Fig. 1). The relatively pure lecithin (dipalmitoleyl-glycerylphosphorylcholine) and lysolecithin (β-monostearoyl-glyceryl-phosphorylcholine) were gifts of Dr. D. J. Hanahan. The faintness of the sphingomyelin spots indicated that only slight amounts were bound at the origin. This was found to be 2% in one analysis in which 1.20 μg sphingomyelin phosphorous were applied. However the intensity of the lecithin origin spots warranted special analysis of the effect of varying the amounts of phospholipid applied.

Table I gives percentage recovery of lecithin at the origin in runs in which varying amounts of lecithin were applied in a mixture containing equal amounts of pure lecithin, sphingomyelin, and lysolecithin phosphorus (P). At least 10%, and as much as 30% of the lecithin was left at the origin when 1.20 μg of lecithin P (29 μg lecithin) or less were applied to the paper. Approximately 7% was left at the origin when 2.40 μg lecithin P (58 μg lecithin) or more were applied.

A means of avoiding this loss at the origin is provided by the technic of "running start" chromatography. Since on regular Whatman #1 filter paper, with the solvents used, all lipid material travels with or close to the solvent front, papers have been prepared so the application of material is made on an unimpregnated area of the filter paper. The applied material travels 2.0-2.5 cm before reaching the silicic acid impregnated portion where separation of the individual phospholipids begins. No Rhodamine G staining material is left at the origin, and separations of individual compounds are favorably affected in shape, so delineation of the boundaries of the spots is more definite. Fig. 2 contrasts the "running start" method with the standard application to silicic acid impregnated paper. The component traveling with an R_f between lecithin and lysolecithin is sphingomyelin. It gives a positive choline test and has the same R_f as pure sphingomyelin prepared from egg yolk by the method of Rhodes and Lea(4). Our recoveries of lecithin have improved 7-10% by the use of "running start" chromatography with loadings up to 5 μg of lecithin P. Repeated applications of extracts may be made, as the variation in the area of loading is not critical. Our recoveries of lysolecithin and sphingomyelin have been essentially the same with or without the "running start" procedure. The technic may have application in other areas where fixation of compounds may occur.

Summary. A technic is described for avoiding loss of lecithin at point of origin during quantitative separation of phospholipids on silicic acid impregnated paper.

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Effect of Drugs on Cx-Protein Responses in the Rabbit.* (26160)

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Availability of appropriate blocking agents commonly provides a valuable tool in studies of sites of action of drugs and sequences of biochemical and physiological reactions. Studies directed toward an elucidation of the role of C-reactive protein, which appears during inflammatory episodes, would be aided if

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blocking agents for formation of C-reactive protein were available. Cortisone and salicylates(1,2) have been reported to be ineffective or only partially effective in this regard while extensive blockade of the reticuloendothelial system with colloidal thorium dioxide (Thorotrast) has been reported to depress the Cx-reactive protein (CxRP) response initially provoked by intravenous injection of Thorotrast in the rabbit(3). In this study a number of substances were screened for their ability to prevent the appearance of CxRP in rabbits. One of the active compounds, fluorometholone, was examined for its ability to block production of CxRP triggered by various stimuli, and some data were obtained with regard to the relationship of CxRP production to antibody formation.

Methods and materials. The hybrid albino male rabbits weighing 2 to 3 kilos were maintained on a diet of Purina rabbit chow and water, *ad libitum*. CxRP antiserum (CxRPA) was obtained from guinea pigs immunized with CxRP isolated from acute phase serum of adjuvant-treated rabbits(4). Samples of blood were obtained from the marginal ear vein of the experimental rabbits before and at various intervals after injection of test materials. Serum CxRP was assayed on dilutions of serum using a capillary precipitin test(5). CxRP levels are reported as the highest dilution giving a positive test with antiserum. To screen various substances for their ability to block the appearance of CxRP, the test substance was suspended in 1 ml of mineral oil and incorporated into 4 ml of ointment base (Aquaphor); and 2.5 ml of the drug-adjuvant mixture injected subcutaneously into pairs of rabbits. CxRP levels in serum were determined 24 hr after injection. Incomplete adjuvant alone elicited a good CxRP response 24 hr after injection and at the same time provided a suitable depot for the test substances. To test the ability of fluorometholone to block CxRP appearance after various stimuli, fluorometholone was injected subcutaneously in a 1% suspension of carboxymethylcellulose in physiological saline, a vehicle which did not usually provoke a CxRP response by itself. The particular stimulus to CxRP production was generally applied immediately after injection of the fluorometholone suspension. Whole body radiation, applied as one stimulus(6) in doses of 250 r, was administered from above and below by 2 X-ray machines operating simultaneously. The technical factors were: 250 kvp, 15 ma; FOD 100 cm; filters 0.21 mm Cu inherent, 0.5 mm Cu parabolic, and 1.0 mm Al; 14 to 17 r/min, measured in air. Seromucoid prepared from 24 hr post-irradiation (500 r, whole body) rabbit serum(7) was applied as another stimulus since it has been found to provoke a CxRP response in contrast to normal seromucoid(8). It was given in doses equivalent to 20 ml of post-irradiation serum. Finally fluorometholone, N-palmitoyl-2-aminoethanol and D,L-*erytho* sphingosine were examined for their ability to block the CxRP response to intravascular formation of antigen-antibody complexes(9). For this purpose rabbit serum from animals immunized with bovine serum albumin (BSA) was fractionated on DEAE cellulose to give the anti-BSA containing γ-globulin (preparation II(9)). A dose of 10 mg of this anti-BSA γ-globulin in 1 cc saline was injected intravenously followed immediately by a solution containing 10 mg of BSA. In experiments on the relationship of CxRP production and antibody production, 1 mg of tobacco mosaic virus (TMV) was injected intravenously into rabbits in which 1 mg/kg of fluorometholone was injected 72, 24 and 4 hr prior to and 4 and 7 hr after injection of TMV. Serum levels of anti-TMV were determined and the level expressed as the highest dilution of serum giving a detectable interfacial precipitation in a ring test with TMV. TMV, employed as an antigen, and as a trigger for CxRP appearance was strain U-2 or U-7 as indicated, which were kindly supplied by Dr. Irving Rappaport. Streptokinase streptodornase (Varidase)[†] was given intravenously in doses of 5000 units. Thorium dioxide as a colloidal 24-26% suspension (Thorotrast)[‡] was injected intrave-

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[†] Obtained from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

[‡] Thorotrast was obtained from Testagar & Co., Inc., Detroit, Mich.

TABLE I. 24 Hour CxRP Response to Various Anti-inflammatory Compounds Incorporated in Adjuvant.

Compound*	Dose/rabbit, mg	24 hr CxRP response†
D,L-*erythro*-sphingosine	7.5	1:4, 1:16, 1:32
D,L-sphingine	7.5	1:4, 1:8, 1:16
Hydrocortisone	10.	1:2, 1:2, 1:4
N-palmitoyl-2-aminoethanol	7.5	neg., neg., 1:4
Methyl prednisolone	2.	neg., 1:1, 1:1
Fluorometholone	1.	neg., neg., 1:1

* Compounds were supplied by Dr. Paul O'Connell of The Upjohn Co.

† All rabbits were negative pre-injection. Responses are recorded for individual rabbits.

nously in doses of 2 cc/kg.

Results. The results of screening various substances for their ability to block a CxRP reaction 24 hours after stimulation with adjuvant are presented in Table I. Adjuvant alone in controls provided a good stimulant of reasonably consistent high serum levels of CxRP. In rabbits treated with adjuvant alone 12 of 15 gave CxRP titers of 1:8 or 1:16 24 hours after injection. The remaining 3 gave responses of 1:1, 1:2 and 1:4. The probability of recognizing appreciable blocking activity is therefore high in tests of a compound in a restricted number of animals in which adjuvant is used as a stimulus for CxRP. D, L-*erythro*-sphingosine and D, L-sphingine displayed no appreciable activity at the levels of drug employed. Hydrocortisone possibly showed some activity, while N - palmitoyl - 2 - aminoethanol and particularly methylprednisolone and fluorometholone showed appreciable activity at dose levels selected. No attempt was made to explore a range of doses for the inactive or weakly active substances since fluorometholone and methylprednisolone evidenced the type of activity we were seeking.

A somewhat higher dose, 0.4-0.5 mg/kg, was chosen in an examination of the CxRP blocking effect of fluorometholone after various stimuli shown in Table II. Fluorometholone at this dose level was ineffective in blocking the response to Thorotrast and to 250 r of whole body irradiation. It was partially effective in blocking the response to injection of 5000 units of Varidase, and was most effective in blocking the response to injection of acute phase seromucoid, TMV, and to the intravascular formation of antigen-antibody complexes.

It was noted that the rate of appearance of anti-TMV was delayed in those rabbits which were challenged with TMV in presence of fluorometholone. Formation of new anti-BSA following immune elimination was also delayed in animals challenged with BSA after passive transfer of anti-BSA. These latter observations indicated that fluorometholone, like cortisone(10), possessed the ability to block or delay antibody formation.

As it has been reported that a rabbit's ability to form high titered antibody correlates with its ability to produce elevated serum levels of CxRP(11), it was of interest to note whether fluorometholone blocked both of these capabilities in parallel.

Rabbits given TMV intravenously responded in 24 hr with a marked CxRP response and had a maximum anti-TMV titer of 1:128 on the 7th day (Fig. 1). Rabbits treated with fluorometholone 72 or 24 hr before or 7 hr after TMV gave maximum anti-

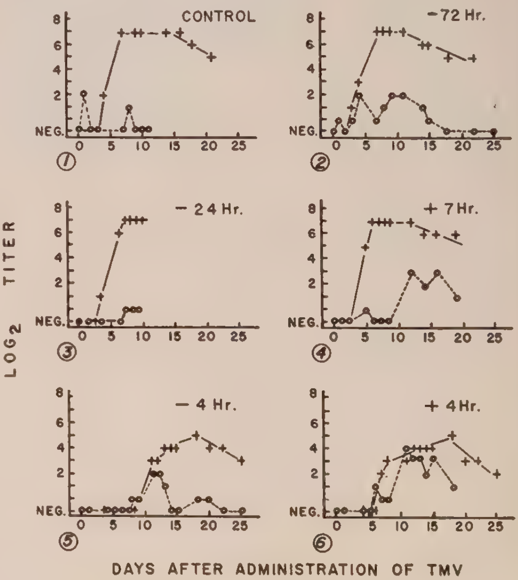

FIG. 1-6. CxRP (○--○) and anti TMV (+—+) titers of rabbits following intrav. inj. of 1 mg of TMV at time 0 and a single intramusc. inj. of 1 mg/kg of fluorometholone at various times before or after TMV. (1) Control given no fluorometholone. (2) Fluorometholone given 72 hr before TMV. (3) Fluorometholone given 24 hr before TMV. (4) Fluorometholone given 7 hr after TMV. (5) Fluorometholone given 4 hr before TMV. (6) Fluorometholone given 4 hr after TMV.

TABLE II. Effect of Agents on CxRP Responses to Various Stimuli.

CxRP stimulus	Drug*	CxRP response 24 hr after stimulation† Control (no drug)	Experimental
250 r whole body irradiation	Fluorometholone	1:8, 1:16, 1:16, 1:16	1:2, 1:4, 1:8, 1:8
Thorotrast	"	1:8, 1:32	1:8, 1:16
Varidase	"	1:2, 1:4, 1:8	neg., 1:1, 1:2, 1:4
Radiation seromucoid	"	1:4, 1:16, 1:32, 1:32	neg., 1:1
Tobacco mosaic virus (strain U-7)	"	1:8, 1:8, 1:16, 1:32	neg., 1:1
Passive intravascular antigen-antibody complex formation	"	1:4, 1:8	neg., neg.
Idem	N-palmitoyl-2-aminoethanol	1:4, 1:16	1:8, 1:8
"	D,L-*erythro*-sphingosine	1:4, 1:16	1:8, 1:8

* Fluorometholone was given subcut. at time of stimulation of CxRP response in a dose of 0.3-0.5 mg/kg. N-palmitoyl-2-aminoethanol was given the same way in a dose of 3 mg/kg 3 hr before and at time of stimulation and D,L-*erythro*-sphingosine was given in a dose of 10 mg/kg 6 hr before and at time of stimulation.

† All rabbits were negative pre-injection. Responses are recorded for individual rabbits.

TMV titers of 1:128 on the 6th or 7th day after TMV, as the controls, but *unaccompanied by a CxRP response* (Fig. 2, 3, 4). Rabbits given fluorometholone 4 hr before or 4 hr after TMV also showed no CxRP response but maximum anti-TMV titer reached was only 1:32 on the 18th day (Fig. 5, 6). The rabbits in which the CxRP response was blocked subsequently showed CxRP responses at a time when the effect of the fluorometholone had worn off.

Discussion. The sphingosines have previously been reported to have anti-inflammatory activity(12,13). It is of interest, therefore, that this was not reflected in the CxRP responses measured under our conditions. In a study by Ganley *et al.*(14) N-palmitoyl-2-aminoethanol exhibited no effect in the cotton pellet and capillary permeability (Evan's blue) tests, although anti-inflammatory activity in Coburn's guinea pig joint anaphylaxis test was noted. In contrast, we noted that N-palmitoyl-2-aminoethanol moderated the CxRP response stimulated by adjuvant but not by intravascular formation of antigen-antibody complexes. The doses used were somewhat higher than those in which they have shown activity in other tests.

Reduction of the acute phase response and attendant disappearance of C-reactive protein has been observed many times clinically in treatment of collagen diseases with ACTH, cortisone, salicylates or aminopyrine. In contrast, cortisone and salicylates have not shown appreciable activity in experiments with rabbits(1,2). The reduction of CxRP levels obtained on stimulation with adjuvant with incorporated hydrocortisone, methyl prednisolone or fluorometholone can probably be attributed to the greater anti-inflammatory activity associated with these drugs(16).

The inability of fluorometholone to block CxRP responses to X-irradiation and Thorotrast in distinction to the activity displayed against other stimuli could be attributed to some difference in site of action on alternate pathways or target organs which each lead to CxRP appearance. It could equally well be attributed to a large difference in the intensity of the inflammatory processes elicited by the various stimuli. Data are not available to support either contention although the latter is now susceptible to experimental test with the availability of a blocking agent. It will be desirable to ascertain this since determination of CxRP responses is relatively rapid and straight forward and could possibly supply a useful adjunct to other methods employed in screening of potential anti-inflammatory compounds. Hill(17) has previously made a similar suggestion.

Wood(11) has noted a useful correlation in that rabbits that produce high levels of CxRP in the serum on immunization with foreign proteins produce good levels of antibody and that rabbits that respond with a poor CxRP response produce smaller amounts of antibody. A possible causal relationship was pointed out. Subsequently Good(15) noted that individuals with either congenital or acquired hypogammaglobulinemia, including the most severe cases, were capable of giving a number of normal acute phase responses, including a C-reactive protein response. He suggested that formation of C-reactive protein is not directly linked with formation and liberation of circulating antibody. In the same vein, we have noted that doses of X-irradiation sufficient to inhibit antibody synthesis do not alter the ability of the rabbit to synthesize CxRP(6). Since we have now found that the CxRP response to injected TMV can be prevented without modifying the course of anti-TMV production as measured in the circulating blood, it would appear that CxRP does not stand in a precursor relationship to circulating antibody or its production.

Two further points may be made from data presented in Fig. 2-6. First from the duration of action of fluorometholone on CxRP and antibody production, it is seen that fluorometholone more effectively blocks Cx RP than antibody formation and that it inhibits antibody production when given as late as 4 hours after antigen, behaving in this regard as does cortisone(10). The lack of CxRP response in one rabbit given the steroid 7 hours after TMV suggests that the process leading to appearance of CxRP can be blocked after many of the events which would otherwise trigger its appearance have already transpired.

Summary. Methyl prednisolone and fluorometholone prevented the appearance of CxRP in rabbit serum following subcutaneous injection of mineral oil-Aquaphor emulsion. Fluorometholone was unable to block CxRP responses to 250 r whole body irradiation or to intravenous injection of Thorotrast or Varidase. It did block CxRP responses to intravenous injection of post irradiation seromucoid or TMV and the intravascular formation of antigen-antibody complexes. Under appropriate time dose conditions fluorometholone inhibited both antibody production and CxRP appearance while under other time dose conditions CxRP appearance was prevented without alteration of antibody production.

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Adrenal Atrophy and Thyroid Inhibition Following PABA.* (26161)

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Atrophy of the adrenal gland following goitrogen administration has been suggested to occur either as a result of a mechanism related to thyroid inhibition or a direct action on the adrenal gland(1). Results of testing various goitrogens showed that all treatments which produced adrenal involution, did so with concomitant thyroid hypertrophy except for administration of p-aminobenzoic acid (PABA)(2). Since the possibility was suggested that PABA treatment influenced the adrenal gland by an extra-thyroidal action (2), this study was undertaken to evaluate thyroid and adrenal gland activities in PABA-fed rats. Since studies(3,4,5) have shown that uptake and incorporation of radioactive phosphorus into the inorganic phosphorus of the adrenal gland vary with conditions affecting the activity of the gland, an attempt was made to assess adrenal activity based on P^{32} uptake. The accumulation of I^{131} in the thyroid gland was taken as an index of glandular activity(6).

Methods. Groups of Sprague-Dawley female rats, average body weights 190 g, were housed 5 to a cage and given ground Purina laboratory chow and tap water *ad libitum.* The experimental groups were fed 2% PABA‡ in the diet for periods of 2, 4 and 12 weeks, and one group was fed 4% PABA in the diet for 12 weeks. Control animals were placed on the ground chow diet alone. Twenty-four hours before termination of an experiment, each rat was given a single intraperitoneal injection of 5 μc of I^{131}. On the next day, 2 hours before killing, each animal was given a single intraperitoneal injection of 100 μc of P^{32}. The animals were killed by decapitation so as to leave the thyroid gland intact. The thyroid and adrenal glands were removed and weighed to the nearest 0.1 mg and the values expressed as milligrams of tissue per 100 g body weight. The thyroid glands were placed in 3 ml of an alkaline KI digestion solution(6) and heated in a boiling water bath until digestion was complete. The adrenal glands were placed in a grinding tube and ground with 5 ml trichloroacetic acid. One aliquot of the adrenal preparation was used for determination of P^{32} and the other was used to determine inorganic phosphorus content(7).

Aliquots from the thyroid and adrenal gland preparations and samples of I^{131} and P^{32} and mixtures of the two, in known concentrations, were counted using a well-type scintillation counter and an end window Geiger-Müller tube. From the data obtained for the standard preparations of I^{131} and P^{32}, simultaneous equations were derived to express the counting ratios of both isotopes in both types of detectors. Using these equations and the counting rates of the tissue samples, radioactivity of I^{131} in the thyroid gland and of P^{32} in the adrenal gland was determined. Level of activity was recorded as counts per minute per gland (c/m/gland) and as counts per minute per 100 mg gland (c/m/100 mg) to indicate total and relative isotope uptake of the tissues.

Results. Feeding rats 2% PABA for 2 weeks failed to produce a significant change in weights of thyroid or adrenal glands (Table I). The thyroid glands from animals given PABA for 4 weeks showed an apparent enlargement when compared to the comparable control group. However, the thyroid gland weights of the 4 week control group

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† Research Participant, Oak Ridge Inst. of Nuclear Studies on leave from Southern Methodist Univ., Dallas, Texas.

‡ Samples of PABA supplied through the courtesy of S. B. Penick Co., Jersey City, N. J.

TABLE I. Effect of PABA Administration on Adrenal and Thyroid Weight of Female Rats.

			Adrenal wt		Thyroid wt	
	No. animals	Body wt, g	mg	mg/100 g body wt	mg	mg/100 g body wt
2 wk treatment						
Control	5	204 ± 7.8	64.5 ± 1.1	31.7 ± 4.9	13.8 ± 3.8	6.8 ± 1.8
PABA 2%	5	213 ± 8.0	58.5 ± 5.7	27.6 ± 3.4	15.8 ± 2.5	7.5 ± 1.4
4 wk treatment						
Control	5	211 ± 7.3	59.0 ± 5.5	27.0 ± 2.3	9.4 ± 1.9	4.5 ± .9
PABA 2%	5	231 ± 19.2	57.0 ± 2.5	24.7 ± 1.8	16.0 ± 3.5*†	6.9 ± 1.2*†
12 wk treatment						
Control	7	246 ± 31.4	55.9 ± 5.3	23.0 ± 3.4	12.1 ± 1.8	5.1 ± 1.1
PABA 2%	7	245 ± 13.2	48.2 ± 5.3*	19.7 ± 2.4	11.8 ± 2.6	4.9 ± 1.2
PABA 4%	8	220 ± 8.1	43.6 ± 6.6*	18.7 ± 3.5*	19.6 ± 7.7*	8.8 ± 3.5*

* $P < 0.05$.

† Significant only in relation to comparable control. Not significant in comparison to other control values.

were somewhat low and the comparison of thyroid glands of 4 weeks PABA treated rats to the 2 week controls revealed no significant difference $(P>0.05)$. The only definite thyroid hypertrophy was seen in the rats administered 4% PABA for 12 weeks where adrenal involution also was evident. In the group given 2% PABA for 12 weeks, absolute adrenal weight was significantly reduced from control weight though relative size of the gland was not different from that of the controls $(P>0.05<0.10)$. Thyroid glands of this latter group were of a size comparable to control glands. In no case was the body weight of a treated group changed from control weights.

The I^{131} level in thyroid glands of PABA-treated rats gave an indication of reduced glandular activity (Table II). While total I^{131} content in thyroid glands from rats given 2% PABA for 2 or 4 weeks was unchanged from the normal, a significant reduction was seen in the isotope uptake per unit gland weight. After the 12 week treatment interval, both experimental groups showed a marked decrease in total and relative thyroidal accumulation of I^{131}. Though adrenal glands from both treated groups showed a reduction in total inorganic phosphorus level, concentration per 100 mg tissue was normal. Only rats fed 4% PABA for 12 weeks were found to have a decrease in P^{32} uptake per

TABLE II. Effect of PABA on Thyroidal I^{131} and Adrenal Inorganic Phosphorus and P^{32} in Female Rats.

	Thyroidal I^{131}		Adrenal inorganic phosphorus		Adrenal P^{32}	
	c/m/gland	c/m/100 mg	μg/gland	μg/100 mg	c/m/gland	c/m/100 mg
	× 10^3					
2 wk treatment						
Control	128 ± 34.9	1006 ± 422.0	23.9 ± 3.9	37.4 ± 4.3	8645 ± 1724	13371 ± 932
PABA 2%	91 ± 18.4	587 ± 159.6*	21.6 ± 3.0	37.0 ± 5.5	8072 ± 1183	13662 ± 2277
4 wk treatment						
Control	123 ± 35.9	1296 ± 252.7	21.4 ± 3.1	35.7 ± 1.9	7907 ± 907	13288 ± 1396
PABA 2%	87 ± 25.2	544 ± 166 *	19.9 ± 2.6	35.0 ± 5.0	11910 ± 5599	21043 ± 10464
12 wk treatment						
Control	226 ± 25.2	1997 ± 40.9	22.2 ± 3.2	40.6 ± 5.3	1150 ± 439†	2094 ± 781
PABA 2%	119 ± 18.2*	1142 ± 42.8*	16.9 ± 3.2*	36.8 ± 2.4	756 ± 403	1670 ± 628
" 4%	113 ± 58.0*	474 ± 81.2*	14.9 ± 1.2*	32.6 ± 2.6	595 ± 106*	1315 ± 334

* $P < 0.05$.

† Conditions required that these samples be counted using a different G-M Counter resulting in a lower counting rate. All samples from this treatment period were counted under the same conditions.

adrenal gland, and in these rats also the level of P^{32} per unit gland weight was unchanged from control values.

Discussion. The possibility of an extrathyroidal action of PABA has previously been suggested since the adrenal atrophy in rats after treatment with 2% PABA was not accompanied by thyroid hypertrophy but was associated with an unusual response of the plasma adrenocorticoid concentrations(2). In comparison, the results reported here indicate that rats fed 2% PABA for 12 weeks show at most only a suggestion of adrenal involution. In these animals, the absolute adrenal weight was reduced but decrease in relative weight was not significant ($P > .05 < .10$). In any event, definite thyroid inhibition was evident in groups treated with the goitrogen for 12 weeks as indicated by the decreased I^{131} uptake in both groups and the thyroid hypertrophy in the animals given 4% PABA. Additional indications of thyroid inhibition were seen in the decrease in I^{131} level in thyroid glands of rats treated for 2 and 4 weeks with PABA. Thus the results indicate that inhibition of the thyroid gland accompanies and precedes adrenal atrophy induced by PABA administration.

Other data fail to indicate a direct action of this goitrogen on the adrenal gland since administration of a single dose of PABA to rats failed to bring about depletion of adrenal ascorbic acid or cholesterol(8). Conditions which affect adrenal gland activity have been associated with changes in incorporation of P^{32} into the inorganic phosphorus of the gland(3,4,5). In the present study, the decrease in inorganic phosphorus and P^{32} in the adrenal glands of PABA-treated rats appears to be related to reduced gland size rather than to changes in activity.

Though the possibility of a direct action of PABA on the adrenal gland cannot be excluded, these data do establish that thyroid inhibition occurs under conditions which produce adrenal involution associated with PABA treatment. Therefore PABA administration could induce adrenal involution by a mechanism associated with thyroid inhibition alone or coupled with a direct action of the adrenal gland(1,2).

Summary. The results clarify a previous report(2) in which the possibility of a direct action of PABA on the adrenal gland was suggested as a more likely mechanism for induction of adrenal atrophy by this goitrogen. These data indicate that under conditions where administration of PABA produced adrenal atrophy, inhibition of the thyroid gland occurred as indicated by reduced thyroidal I^{131} uptake. Thus further evidence is presented to associate goitrogenic activity with ability to induce adrenal atrophy, though the possibility of a direct action of PABA on the adrenal gland coupled with thyroid inhibition can not be excluded.

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Hydroxocobalamin: Physiological Retention in the Dog. (26162)

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Therapy aimed at maintained elevation of Vit. B_{12} in the body is hampered by the rapid urinary excretion of the vitamin and consequent need for repeated administration. The therapeutic effectiveness of a single injection of a small amount of cyanocobalamin in pernicious anemia, with rapid excretion in the urine of larger amounts has been established(1). Indeed, Reisner and Weiner(2) state that individual doses of Vit. B_{12} above 50 μg are not warranted since they exceed the renal threshold value, are rapidly and quantitatively excreted, and do not prolong the period of remission. Mollin and Ross(3) find that hemapoiesis is normoblastic when serum Vit. B_{12} levels are maintained at 100 μg or more, but that megaloblasts appear in the marrow of pernicious anemia patients when serum levels fall below that figure. In the opinion of Mollin and Ross, tissue Vit. B_{12} deficiency must be extreme before serum levels fall below normal and megaloblastic anemia develops. To restore the depleted tissues to their normal range of 1 to 2 mg, considerably more Vit. B_{12} is needed than for producing hematological remission and they suggest that 5-1 mg doses, administered over a 2-week period, with retention of 20 to 30% of each injection would constitute rational initial therapy, followed by maintenance with monthly injections of 100 μg. This constitutes a more intensive therapy than recommended by Unglaub and Goldsmith(4), but is supported by reports(5,6) that therapy adequate to produce a hematological remission may be inadequate for reversing the neurological disease pattern in the Vit. B_{12} deficient individual.

Apart from its physiological role in maintaining normal metabolism, Vit. B_{12} has been reported to exert a pharmacological effect in a variety of conditions such as non-specific neurological disease and neuroblastoma in infants(2,7,8) when massive doses, 1 mg or more, are administered repeatedly. Thus, it appears that there would be a therapeutic advantage in certain disease states to maintaining an elevation of the Vit. B_{12} circulating in the body. To this end considerable effort has been directed toward the search for forms of Vit. B_{12} that upon injection would be better retained by the body and maintain elevation of blood levels for a longer period of time. This effort has met with some success in development of effective depot forms of cyanocobalamin(9,10).

We are reporting in this paper that high and prolonged serum levels are obtained in dogs after injection of aqueous solutions of hydroxocobalamin.* Since this substance is injected in true solution its retention must be due to other factors than a depot effect and, therefore, the term physiological retention has been used to describe this phenomenon

Experimental procedure. Dogs were individually caged and fed standard rations throughout the experiment. They were selected at random from animals being held for future pharmacologic study and, except for those which had received antibiotic therapy for infections, had not been on previous medication. Since this study was concerned with maintenance of elevation of serum Vit. B_{12} levels, samples were not collected until 5 hours post injection, when preliminary investigation had revealed that the levels following administration of aqueous cyanocobalamin had passed their peak and maximum rate of decline.

Solutions of the cobalamins to which 0.9% benzyl alcohol had been added as a preservative were sterilized by filtration and injected into the gluteal muscle (500 μg). Blood was obtained by venipuncture of the jugular vein

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* Hydroxocobalamin has been referred to in the literature as vit. B_{12a} and vit. B_{12b}(11). According to Smith(12) it exists as aquocobalamin except in alkaline solutions.

TABLE I. Comparison of Effect of Single Injections of Hydroxocobalamin (H)* and Cyanocobalamin (C) on Vit. B_{12} Serum Levels of the Dog.

		mμg vit. B_{12} activity/ml											
		Hr after administration											
	No. dogs	0		5		24		48		96		168	
Exp.	compared	H	C	H	C	H	C	H	C	H	C	H	C
1	2	.60	.61	15.70	13.20	3.98	1.32	1.86	.91				
2	2	.33	.42	18.60	3.31	4.68	1.41	2.51	.84	1.06	.79		
3	2	.32	.47	19.10	8.51	4.22	1.60	2.54	1.43				
4	2	.28	.32	12.60	4.03	2.69	1.10						
5	2	.23	.26	34.30	3.31	4.52	1.41	1.93	.85	1.16	.62		
6	3	.16	.33	31.80	5.66	6.76	2.14	2.84	1.56	1.51	.94	.61	.52
6A†	3	.20		30.85		5.37		2.88		1.55		.57	
7	2	.29	.26	72.40	4.22	10.10	1.27	4.22	.78			1.26	.44
Avg		.28	.36	25.10	5.33	4.98	1.48	2.57	1.05	1.27	.79	.78	.48
P value				<0.05		$<.001$		$<.001$		$<.025$		NS	

500 μg of each preparation were inj. into the gluteal muscle of 2 (3) dogs per experiment. Blood was obtained for serum analysis at indicated interval. Values are expressed as geometric mean.

* Hydroxocobalamin in Exp. 1 through 4 analysed as 75% of total cobalamins. Purity of hydroxocobalamin in Exp. 5-7 was reported to be 90%.

† These data not included in the statistical analyses.

0, 5, 24 and 48 hours post injection. Serum was drawn off after clot formation and kept frozen until assayed.

Vit. B_{12} was determined by the microbiological assay procedure employing *L. leichmannii,* ATCC 4797(13) with the following modifications:

(1) Double strength assay medium was supplemented with 200 mg/liter each of guanosine, guanylic acid and calcium chloride, and 1 g of DL-α-alanine. The final mixture was adjusted to pH 5.8.

(2) *L. leichmannii* was carried in single strength basal medium supplemented with 0.1 mμg per ml of Vit. B_{12} for several 24-hour transfers prior to use as an inoculum, returning to stock milk culture every 2 weeks. The inoculum was prepared by washing the cells 3 times with sterile physiological saline, resuspending in saline, and diluting 1 to 5,000.

(3) Cyanocobalamin was used as the standard in the range from 10 to 100 μμg per 10 ml single strength medium. Hydroxocobalamin was equal to cyanocobalamin in microbiological activity.

Serum samples were diluted with water and assayed without further treatment. Because of serum protein precipitation in the assay tubes, assays were read titrimetrically instead of turbidimetrically after 72 hours incubation at 37.5°. All glassware employed, both in collection of blood samples and in the assay, was meticulously cleaned.

Results. A comparison of serum Vit. B_{12} activity after injection of 500 μg doses of cyanocobalamin and hydroxocobalamin is shown in Table I. All sera in a given experiment were assayed simultaneously. Although an equivalent total cobalamin dose was administered, the hydroxocobalamin samples were not chemically pure, so that in fact the first 4 experiments compare an actual hydroxocobalamin concentration of 375 and the last three 450 μg with the 500 μg dose of cyanocobalamin. Regardless of this discrepancy, the serum cobalamin concentrations following administration of hydroxocobalamin are consistently significantly higher than those following administration of cyanocobalamin. Hydroxocobalamin was administered in a stabilized solution in Exp. 5, 6 and 7. The data shown in Exp. 6A were obtained simultaneously with those in Exp. 6 on dogs injected with the same concentration of hydroxocobalamin in water and show that the stabilizing formula does not alter the inherent activity of the hydroxocobalamin.

The prolonged serum concentration observed after injection of hydroxocobalamin suggested that, in comparison with cyanocobalamin, it is more readily taken up by body receptor sites. It was therefore of interest to

TABLE II. Comparison of Total and Bound Vitamin B_{12} in Serum after Administration of Cyanocobalamin or Hydroxocobalamin to Dogs.

Hr after admin.	mμg vit. B_{12}/ml			
	Hydroxocobalamin		Cyanocobalamin	
	Total	Bound	Total	Bound
0	.264	.160	.305	.165
5	20.6	6.25	8.81	1.05
24	5.28	1.60	1.80	.638
48	1.61	.852	.782	.590

500 μg of each preparation were inj. into gluteal muscle of 2 dogs. Avg values are given.

determine whether there was a difference in amount of bound Vit. B_{12} activity in the serum of dogs injected with the 2 cobalamin analogs. Using the charcoal absorption technic of Miller(14) to remove free Vit. B_{12}, the sera from dogs in one study group were assayed before (total) and after (bound) norit treatment. The results are shown in Table II. The amount of bound Vit. B_{12} in the serum of dogs injected with hydroxocobalamin was greater than in dogs receiving cyanocobalamin. Preliminary studies had established that the concentration of charcoal employed was adequate to remove either of the free cobalamins up to 50 mμg.

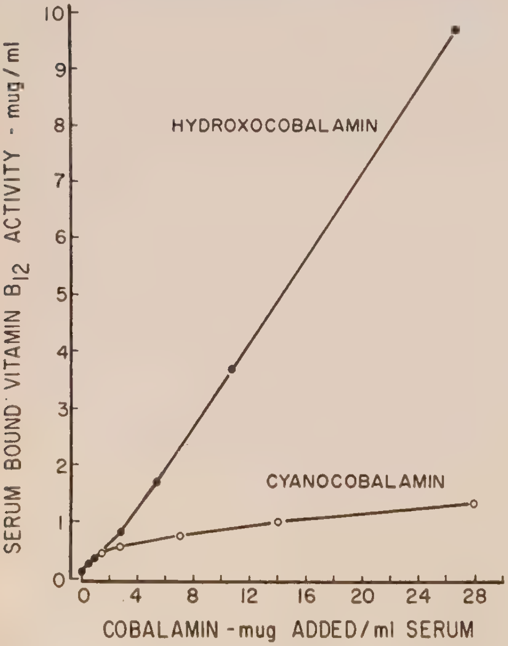

FIG. 1. Binding of cobalamins by normal dog serum.

The difference in binding capacity of dog sera for the 2 cobalamins was clearly demonstrable *in vitro*. Fig. 1 shows that when serum was incubated with varying concentrations of hydroxocobalamin and subsequently treated with charcoal to remove free cobalamin, the concentration of bound Vit. B_{12}, as determined microbiologically, increased in proportion to the original concentration to which the serum was exposed. On the other hand, serum binding of cyanocobalamin was proportionately less as concentration was increased, as Miller had found, and appeared to level off at a relatively low concentration. A possible explanation for the difference in serum binding may lie in the chemical stability of cyanocobalamin which limits the sites at which it may be bound as compared to hydroxocobalamin which readily dissociates and may equilibrate with a variety of serum constituents.

Discussion. Most of the cobalamin found in tissue is known to be bound to protein and a large part of it is present in the noncyanide form(15). The data presented here suggest that hydroxocobalamin may be more available to tissue receptor sites than cyanocobalamin. By whatever mechanism it is retained for longer periods in the circulating blood, the time interval for effective tissue saturation is increased. If the amounts bound in serum are indicative, tissue binding generally may be increased. Preliminary clinical studies(16) in normal subjects indicate that this may be the case, since there appears to be in man slower disappearance of hydroxocobalamin from site of injection, increased liver uptake and less rapid urinary excretion when compared with cyanocobalamin. Thus while hydroxocobalamin is not a "depot" compound in the usual sense of the word, it appears to have physiological "depot" activity.

It has long since been established that hydroxocobalamin is as effective as cyanocobalamin in treatment of pernicious anemia (17). If there is any virtue to the supposition that prolonging the availability of cobalamin will be of therapeutic advantage in treatment of certain neurological disease, hydroxocobalamin may prove more effective in these conditions than cyanocobalamin.

Summary. Hydroxocobalamin, when injected intramuscularly into dogs, produces more prolonged elevation of serum Vit. B_{12} than does injection of equal quantities of cyanocobalamin. The possible physiological significance of this observation is discussed.

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Bile Salt Absorption at Various Levels of Rat Small Intestine.* (26163)

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Frolicher(1), using an *in vivo* technic, showed that the terminal ileum of the rat has the ability to absorb cholates more rapidly than does the upper jejunum. The upper jejunum, in turn, was shown to be a better cholate absorber than the duodenum. The present investigation confirms and extends Frolicher's findings.

Methods. The experiments were performed on adult, male, albino rats (Holtzman Co.) which had been fasted for 24 hours with access to tap water. Extract of Ox Bile, N. F. (Wilson Laboratories) was used as a source of conjugated cholate. The bile salts in this preparation consist principally of sodium glycocholate and sodium taurocholate. An approximately 1.0% solution of Extract of Ox Bile in distilled water was used as the initial test solution. The cholate concentration of this solution in the various experiments ranged from 11.6 to 13.2 μmoles per ml.

An *in vivo* isolated loop technic was used. An attempt was made to overcome two major problems often encountered in such technics: (a) the progressive decrease in intralumenal hydrostatic pressure, and (b) the difficulty in completely removing all of the remaining test solution from the loop at end of experiment.

Each animal was anesthetized with sodium pentobarbital (about 40 mg/kg, I.P.); its abdomen was opened in the midline; and a segment of small intestine, roughly 10 to 15 cm long was cannulated at both ends. The position of the segment along the intestine varied from animal to animal. The bile duct was tied off if it entered the loop of intestine selected. The cannulae were connected

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through 3-way stopcocks to two 10 ml syringes ("A" and "B"). The abdomen was closed and the segment rinsed with three 10 ml portions of 0.9% NaCl from syringe A, then emptied by flushing with air. The segment was then rinsed from syringe A with two 10 ml portions of the same bile salt solution that would subsequently be used in the test period (this was called the "conditioning rinse"), and flushed with air until no further fluid could be removed by passage of air through the loop. A 10.0 ml portion of test solution was then placed in syringe A and allowed to infuse into the segment and up into the barrel of syringe B (without plunger) to a height of about 4 cm above the tips of the cannulae. About 4 ml of solution remained in syringe A.

During the test period syringe barrel B was left open to the loop, thus serving as a reservoir. The intralumenal hydrostatic pressure was maintained within narrow limits by addition of test solution to this reservoir. After a one hour test period the system was drained from the stopcock on syringe B and flushed with air. The amount of cholate remaining in the system was expected to be quite close to the amount present in the system before the test period began (as a result of the conditioning rinse), thus eliminating the necessity of attempting to remove all of the residual solution at the end of test period by means of a series of rinses.

To test this contention a series of immediate recovery experiments were performed. After the conditioning rinse was removed, 10.00 ml of test solution were placed in the system, allowed to rise into syringe B in the usual manner, then promptly removed from the system without allowing appreciable time for absorption to occur. Mean recovery from 4 animals was 98.2% with a standard error of ± 0.15%. This recovery was considered to be satisfactory.

The volume of recovered solution was measured to the nearest 0.1 ml and cholate concentration determined by the photometric method of Irvin, Johnston, and Kopala(2). Amount of cholate absorbed was calculated by subtracting the amount recovered after the test period from that present in the 10.0 ml of test solution used (plus amount present in any additional test solution used to maintain the hydrostatic pressure).

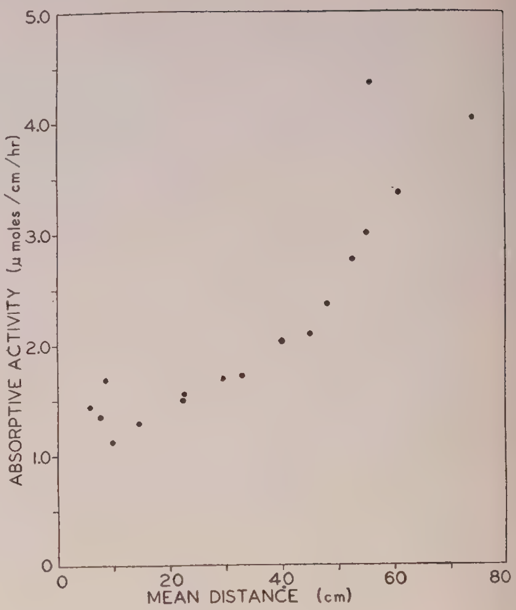

FIG. 1. Absorptive activity for cholate as a function of distance from pylorus.

After the experiment the small intestine including the loop studied was excised, placed in a metal trough filled with Ringer's solution at 20 to 25°C, gently straightened without stretching, and floated alongside a centimeter scale. In this way length of the loop, and distance from pylorus to middle of the loop (mean distance) were measured.

Results. The results obtained in 17 animals are shown in Fig. 1. In this figure absorptive activity, defined as μmoles of cholate absorbed per cm of intestine per hour, is plotted against the mean distance of the segment from the pylorus. Absorptive activity for cholate increased as the intestine was descended. The slope of the best fitting straight line through the points was significantly greater than zero at the 0.1% level of confidence. However, absorptive activity appeared to be an exponential function of mean distance.

Discussion. The absorption of bile salt is a more important function of the intestine than might immediately be apparent. A large percentage of the secreted cholate must be reabsorbed by the intestine to maintain the cholate level in the enterohepatic circuit requisite to normal lipid absorption. The observed pattern of absorptive activity along the intestine would seem to be ideally suited to the dual function of keeping the cholates in the lumen to promote lipid absorption for as long as possible and yet allowing their eventual recovery to the animal.

The existence of such a gradient in absorptive activity indirectly supports the idea that some specific system exists in the mucosal epithelium to absorb cholate. The magnitude of the cholate gradient becomes more striking when one considers that the mucosal surface area per unit length of small intestine decreases with distance from the pylorus(3).

It is interesting to note that this gradient for bile salt *in vivo* is oriented in the opposite direction to the absorptive activity gradients for glucose along the small intestine of the rat(4), and for palmitic acid along the hamster small intestine(5) observed in experiments with surviving small intestine *in vitro*.

Kremen, *et al.*(6) have shown in dogs that a much greater defect in fat absorption occurs when the distal half of the small intestine is resected than when the proximal half (below the duodenum) is removed. The possibility should be considered that the marked fat absorption defect observed by these authors after distal resection resulted from elimination of a highly active area of bile salt absorption with a consequent decrease in amount of cholate in the enterohepatic circuit, and did not necessarily reflect a greater inherent ability of the distal small intestine to absorb fat.

Summary. Using an *in vivo*, acute loop technic it has been shown in the rat that absorptive activity for conjugated cholate increases nearly 4-fold as the small intestine is descended from duodenum to terminal ileum.

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Pyridoxine Deficiency in Hyperthyroidism.* (26164)

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Hyperthyroidism is associated with an increased requirement not only of calories, but also of essential micronutrients. When they are not provided in the daily dietary, or fail to be utilized, weight loss and nutritional deficiencies may ensue.

Among the main micronutrients whose quantitative requirements are elevated in experimental hyperthyroidism are certain of the B complex vitamins. Thus, Cowgill and Palmieri concluded from experimentally produced thyrotoxicosis that there is some relation between amount of B requirement for maintainance of weight and metabolism of the organism(1). In view of the fact that B-6 is involved in a great many enzymatic reactions, it seemed advantageous to investigate pyridoxine metabolism in hyperthyroidism in man.

Material and methods. The presence of B-6 complex is required for metabolic breakdown of tryptophan. In induced B-6 deficiency, derangement of tryptophan metabolism is manifested by increased excretion of xanthurenic acid after a tryptophan load(2). Administration of B-6 corrects this aberration

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* We are indebted to Dr. H. Menduke, Jefferson Med. College, for statistical evaluation of data.

TABLE I. Xanthurenic Acid Excretion in Patients with Hyperthyroidism.

			Before tryptophan			After tryptophan			After tryptophan + B6		
Case No.	Sex	Age	Vol	Creat.	Xa.	Vol	Creat.	Xa.	Vol	Creat.	Xa.
1	♀	42	1250	1.1	3	900	1.1	11	1200	1.1	11
2	♀	39	1210	.6	10	1110	.6	33	1310	.7	12
3	♀	25	350	.4	2	770	.7	31	500	.4	8
4	♀	22	1220	.7	6	1140	.6	105	1490	.7	16
5	♀	50	1270	1.0	7	970	.7	24	1080	.8	4
6	♀	48	2340	.8	1	2190	.8	195	900	.7	14
7	♀	23	2250	.8	22	3250	1.0	150	680	.6	7
8	♀	66	740	.54	6	890	.87	62	1225	.7	15
9	♀	48	540	.4	2	670	.5	67	500	.4	11
10	♀	50	2370	.6	6	1920	.4	45	1300	.5	6
11	♂	55	2330	1.1	3	2130	1.3	19	1920	.9	7
12	♀	40	1075	.9	20	1260	.9	80	1670	1.1	20
13	♀	20	1400	.9	12	1260	.6	141	2400	1.0	39
14	♀	50	430	.5	2	500	.5	44	800	.8	15

Vol = Volume in ml. Creat. = Creatinine in g/24 hr. Xa. = Xanthurenic acid in mg/24 hr.

and xanthurenic acid excretion is greatly decreased or becomes normal(3). We have used the increased excretion of xanthurenic acid (XA) after a load test of tryptophan and its correction by administration of pyridoxine as an index of a relative pyridoxine deficiency. Fourteen patients (13 females and 1 male) were studied from the medical services of the Hahnemann Med. College and Hospital and the Philadelphia General Hosp.† There was no selection other than contingent on established clinical criteria of thyrotoxicosis, such as weight loss, tachycardia, easy fatigability, thyroid enlargement and laboratory findings consistent with the disease, *i.e.*, increased PBI and I^{131} uptake, and elevated metabolic rate, and rapid I^{131} conversion (Table II).

Controls. Fourteen patients (9 females, 5 males) of similar age, free from hyperthyroidism, served as controls. Both groups of patients were instructed to consume a liberal regular house diet (average intake of 3000 calories); to our knowledge they were not taking any vitamin supplements, but were given 10 mg of Thiamine and 5 mg of Riboflavin daily during the study, since coenzymes containing these vitamins are known to be involved in tryptophan metabolism. Xanthurenic acid excretion was determined by a modified method of Rosen(4) on a 24 hr urine specimen. A control xanthurenic acid excretion before the tryptophan load test was done. On the morning of the second day, a 10 g dose of D, L-tryptophan (Dow Chemical Co.) was given mixed in fruit juice and a second 24 hr urine collection obtained. On the morning of the third day, 50 mg of pyridoxine-HCl (U.S.P.) was administered intramuscularly 30 minutes before a second dose of 10 g of tryptophan was given, followed by the third and final 24 hr urine collection. Urine specimens were collected under toluence, and after the reaction was adjusted to pH 5.5 with 5 normal hydrochloric acid, aliquots were stored in amber glass bottles at 4°C. Accuracy of collections was checked by carrying out urinary creatinine determinations by the method of Folin(5). When a variation in creatinine of more than 25% was found, the patient was dropped from the study. Complete collections were deemed essential, since studies had revealed that the largest amounts of xanthurenic acid excretion appeared in the first 8 hours after oral ingestion of tryptophan. None of the patients taking tryptophan experienced any severe side reactions. No change in blood urea nitrogen was noted in any of the patients during the study. Serum glutamic-oxaloacetic transaminase determinations were carried out by the method of Cabaud *et al.*

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† We are indebted to Drs. Charles Shuman and Stephen Dubell, Temple Univ. Hospital, Philadelphia, Pa., for permission to include 2 hyperthyroid patients studied for pyridoxine deficiency. This work has been supported in part by a grant from HEW, Public Health Service.

TABLE II. Parameters of Thyroid Function.

Case No.	I^{131}, %	PBI	Conversion ratio, %	Basal metabolic rate
1	61	14	81	+46
2*	90	12	27	+30
3	58	12	52	+64
4	50	11	—	+47
5	95	21	89	+35
6	92	20	77	+60
7	84	15	—	+59
8	47	7	56	—
9	59	17	61	—
10	65	20	91	+59
11	84	—	91	+72
12	54	9	—	—
13	—	22	—	+41
14	47	8	61	—

Normal laboratory values: I^{131} uptake, 10 to 40%; protein-bound iodine (PBI), 3.5-7.5 γ %; I^{131} conversion ratio, 13 to 42%; basal metabolic rate, minus 10 to plus 10.

* Low conversion ratio in case 2 is due to cessation of propylthiouracil therapy 2 wk prior to I^{131} studies.

(6) in 6 patients, since the enzyme is known to be involved in pyridoxine metabolism. The significance of transaminase determinations as an index of pyridoxine deficiency will be described elsewhere jointly with transaminase results in cardiac failure(7). None of the present subjects showed evidence of heart failure, as determined by the usual clinical criteria.

Results. Tables I and III present results of xanthurenic acid excretion, creatinine, and volume of 24 hr urine collections on all subjects. Fourteen hyperthyroid patients and 14 control patients were selected for analysis who fulfilled the following criteria: 1. There was a pre-tryptophan, a post-tryptophan, and a post-tryptophan and pyridoxine value. 2. Level of creatinine in all urine collections was 0.4 g per day or higher. Collections with levels of 0.3 g or less were considered as fair evidence of poor collection of urine and were discarded. Means and standard deviations for these subjects are shown in Fig. 1.

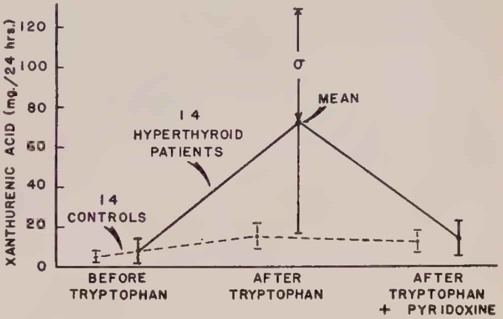

FIG. 1. Response of hyperthyroid and of control patients to tryptophan load and to tryptophan load after pyridoxine.

The hyperthyroid groups and control groups were quite comparable with respect to both mean and standard deviation in pre-tryptophan level of xanthurenic acid excre-

TABLE III. Non-thyroid Patients.

Case No.	Sex	Age	Control			AT-1			AT-B6			Diagnosis
			Vol	Creat.	Xa.	Vol	Creat.	Xa.	Vol	Creat.	Xa.	
1	♀	32	3000	1.0	8	1750	.9	13	1350	.8	13	Rheumatic heart
2	♀	47	1100	1.1	1	1095	1.1	9	855	1.0	7	Melancholia
3	♂	19	900	.64	3	1740	1.71	10	1300	1.18	8	Fractured femur
4	♀	60	3100	1.5	5	3260	1.4	20	2600	1.4	15	Diabetes mellitus
5	♂	22	1400	1.59	4	570	1.14	8	1540	1.51	9	Anxiety neurosis
6	♂	34	910	1.2	1	1750	1.5	22	1340	1.0	11	Duodenal ulcer
7	♀	42	1200	.89	9	2350	1.09	27	2940	1.09	20	Hypochronic anemia, unknown etiology
8	♀	37	3085	1.8	9	1965	1.9	19	3010	1.8	20	Diabetes mellitus
9	♀	62	1985	1.5	8	2089	1.6	18	2100	1.6	18	Arteriosclerotic heart disease
10	♀	30	700	.86	9	1560	.93	14	1310	1.24	12	Hyperventilation syndrome
11	♂	25	1185	1.8	3	1745	1.4	6	680	1.1	1	Pulmonary osteoarthropathy
12	♀	26	830	1.0	1	810	1.1	18	560	1.1	16	Psychoneurosis
13	♀	60	2970	2.3	2	2210	1.3	11	3200	1.9	12	Diabetes mellitus
14	♂	44	1090	1.6	8	2550	1.5	20	1800	1.5	12	Chronic osteomyelitis

Vol = Volume in ml. Creat. = Creatinine in g/24 hr. Xa. = Xanthurenic acid in mg/24 hr.

tion with means of 7.3 and 5.1 mg/24 hr respectively. Following tryptophan load, each showed a highly significant mean increase over pre-tryptophan level (probability of "t" less than 0.001 in each case). The mean increase for hyperthyroid group was, however, significantly greater than that for the control group (probability of "t" less than 0.01) so that the former reached a significantly higher mean level of excretion (71.0 *vs.* only 15.4 mg/24 hr). In addition, the variance for the hyperthyroid group was considerable and significantly greater than that of the control group (standard deviation of 56.0 and 6.1 mg/24 hr respectively). This greater variance in the hyperthyroid group was to be expected because these patients differed greatly in severity of metabolic derangement, while the control group was more homogenous in that none suffered from hyperthyroidism. The highest level attained by a control patient was 27 mg/24 hr, while four-fifths of the hyperthyroid patients exceeded this value.

When a tryptophan load was given after pyridoxine, the level was, for each group, significantly lower than with tryptophan load alone but significantly higher than in the pre-tryptophan period. In 17 of the 28 subjects, this pattern (lowest in the pre-tryptophan, highest in the post-tryptophan and intermediate in the post-tryptophan plus pyridoxine period) was followed exactly. The hyperthyroid group, which had attained a higher level than the control group in the post-tryptophan period, showed a greater decline from post-tryptophan to post-tryptophan plus pyridoxine so that the 2 groups were at approximately the same level after pyridoxine (13.2 mg/24 hr for the hyperthyroid group and 12.5 mg/25 hr for the control group). They also showed comparable variances after pyridoxine (standard deviation of 8.7 and 5.2 respectively. While the level after tryptophan plus pyridoxine was higher than that in the pre-tryptophan period, the difference was significant only in a statistical sense; however, it is apparent that pyridoxine decreased xanthurenic acid excretion levels comparable to those of the control pyridoxine treated group (Fig. 1).

Discussion. The statistically significant high levels of xanthurenic acid in patients with hyperthyroidism and the improvement in derangement of tryptophan metabolism with a resultant return to decidedly lower levels of xanthurenic acid upon administration of pyridoxine, tend to indicate that the nutritional status of hyperthyroid patients with respect to pyridoxine is subnormal. The only reference in the literature in regard to tryptophan metabolism in hyperthyroidism is that by Wachstein and Lobel(8). In their series of 44 patients with various diseases, studied with the tryptophan load test, 3 patients with hyperthyroidism had 24 hr urine xanthurenic acid excretion of 84 mg, 33 mg and 31 mg respectively. After administration of a total of 50 mg pyridoxine to 2 patients and 100 mg pyridoxine to a third patient, xanthurenic acid excretion after tryptophan load was zero. The patient who originally excreted 84 mg xanthurenic acid had a third tryptophan load test, but no pyridoxine, 3 weeks later the results of the xanthurenic acid excretion were considerably higher than in the first test (128 mg). It is to be regretted that no information is given as to the patient's exact clinical status and thyroid function parameters at a time when xanthurenic acid excretion was the highest (128 mg). The question arises as to the mechanism responsible for the deranged tryptophan metabolism in hyperthyroidism. Calculated values of daily intake of B-6 in the subjects under our study were 2.0 to 2.5 mg.‡ Daily requirement for normal human adults is estimated to be from 1 to 2 mg (Nat. Research Council, 1958). It is apparent that an oral intake of B-6 considered adequate for a normal person is totally inadequate for a patient stressed with hyperthyroidism. An analagous situation with regard to thiamine exists in the disease(9). Another factor which may contribute to abnormal tryptophan metabolism may be failure of B-6 to be incorporated in the enzyme system required for normal tryptophan metabolism. However, when pyridoxine was administered intramuscularly to our patients, bio-chemical response to tryptophan was normal, tending to indicate that availability of B-6 was probably the limiting factor for normal tryptophan metabolism in our patients with hyperthyroidism. The patients in this study showed no overt clinical manifestations of pyridoxine deficiency. However, biochemical changes, particularly an abnormal tryptophan load test, become demonstrable before symptoms manifest themselves in experimentally induced human pyridoxine deficiency(10,14).

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‡ Values for B-6 were calculated from Tables of Richard W. Vilter: Wohl and Goodhard, *Modern Nutrition in Health and Disease*, 1960, p. 349, Lea and Febiger.

One of the patients with hyperthyroidism showed obvious signs of muscle weakness while under study; daily treatment with pyridoxine without other antithyroid therapy resulted in considerable gain in muscle strength. Based on Spies' work on the influence of pyridoxine intravenously on muscle weakness in pellagrins(12), Rosenberg *et al.* (11) administered B-6 to a group of patients who suffered extreme muscle weakness and exhaustion, in whom no other symptoms of B Complex deficiency were evident. Among this group were 3 patients with hyperthyroidism who received pyridoxine intravenously, with no anti-thyroid therapy. As a result of this procedure, they noted increased ability to perform work.

It is reasonable to infer that some symptoms, *e.g.*, muscle weakness in hyperthyroidism, may not be due directly to excessive activity of the thyroid hormone, but rather reflect a conditioned manifestation of pyridoxine deficiency, not unlike thiamine(9,13) and nicotinic acid deficiency(15). More extensive studies on the chemical changes at the cellular level of biopsied muscle from hyperthyroid patients may advance our knowledge of the causal relationship between pyridoxine deficiency and muscle weakness in hyperthyroidism.

Summary. The occurrence of pyridoxine deficiency was determined in 14 patients with hyperthyroidism and 14 control euthyroid patients by the tryptophan load test. Urinary xanthurenic acid excretion following the tryptophan load test was significantly greater in hyperthyroid patients than in controls. Mean excretion of xanthurenic acid in hyperthyroid cases was 71.9 mg/24 hr with standard deviation of 56.0. Mean xanthurenic acid excretion of control cases was 15.4 with standard deviation of 6.1. After pyridoxine administration, mean xanthurenic acid excretion was 13.2 with standard deviation of 8.7 in the hyperthyroid cases, while in the control group, xanthurenic acid excretion was 12.5 with standard deviation of 5.2. Since pyridoxine is required for orderly catabolism of tryptophan and pyridoxine corrected this metabolic aberration, it is not unreasonable to state that in hyperthyroidism, the availability of pyridoxine is limited. Until the actual significance of these findings becomes more clearly defined, it would seem reasonable to suggest that use of pyridoxine in addition to anti-thyroid medication may prove of distinct benefit in treatment of the hyperthyroid patients.

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Spinal Cord and Adrenocorticotropin Release.* (26165)

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Earlier investigations have provided evidence that adrenocorticotropin (ACTH) release is under control of one or more chemical mediators delivered to adenohypophyseal tissue *via* the hypophyseal portal venous system. Assuming this to be the case, the location of site or sites of origin and the mechanism controlling release of these chemical mediators, often referred to as corticotropin releasing factors (CRFs), becomes important. The hypothalamus, and in particular the median eminence, has been proposed by several workers as the site of release. However, sites of origin of CRFs located more peripherally have also been proposed. These proposals have been made on the basis of experimentally induced increases in ACTH release following stimulation of denervated structures. To account for the increase, blood borne chemical mediators such as epinephrine, or products of tissue destruction, are considered to be carried from a peripheral site to the pituitary *via* systemic circulation(1,2,3,4,5).

The apparently conflicting experimental results, methodological shortcomings and deficiency of adequate controls of previous experiments in cord-sectioned animals prompted a reinvestigation of the problem. The observations reported here offer no support for a peripherally elaborated CRF since application of stimuli below the level of cord section failed to increase rate of release of ACTH.

Methods. Adult male rats (Sprague-Dawley) weighing 225-275 g, were used following one to 2 weeks acclimatization in this laboratory. The ACTH-releasing effect of a given procedure was evaluated by means of adrenal ascorbic acid depletion(6). The rats were sacrificed by application of a large surgical forceps at the level of the neck. This procedure decapitated the rat and prevented exchange of blood between head and trunk. The left and right adrenal glands were removed and ascorbic acid concentrations were determined separately, averaged, and expressed in milligrams adrenal ascorbic acid per 100 g fresh adrenal tissue. The assumption is made in these experiments that changes in adrenal ascorbic acid concentration of the experimental animal reflect increased rate of release of ACTH from the adenohypophysis.

Adrenal ascorbic acid depletion was studied in unanesthetized intact and in unanesthetized cord-sectioned rats after electrical stimulation and after injection of U.S.P. Corticotropin Reference Standard. Cord-sectioned rats were prepared under ether anesthesia. The spinal cord was sectioned at T_2 and a short probe destroyed spinal segments T_1 to T_3. The operated rats were caged individually in a room regulated at 30°C ± 1.0.

Unanesthetized rats were prepared for electrical stimulation by placing electrodes consisting of a length of soft copper wire either on both hindpaws or on both forepaws. An electrical stimulus believed sufficient to activate all nerve fibers in the vicinity of the electrodes was applied. The stimulation consisted of a square wave current flow having peak value of 4 to 7 milliamperes and a pulse duration of 3.2 milliseconds delivered at 100 pulses per second. The square wave current was turned on for 10 seconds, then off for 20 seconds, by a timer for a total of 12 minutes. Current flow was monitored on a cathode ray oscilloscope.

Results. Effect of spinal cord transection on adrenal ascorbic acid concentration and on adrenal weight. Adrenal ascorbic acid concentrations were determined at 4, 24, and 48 hours and at 7 days after operation (Table I). Four hours after spinal cord transection, adrenal ascorbic acid concentration was less than normal. At 24 hours, concentration was higher but still less than normal. At 48 hours, concentration was not significantly different from that of normal animals receiving

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TABLE I. Adrenal Ascorbic Acid Concentration after Spinal Cord Transection.

Time after cord transection	
Intact rats*	450 ± 9 (7)†
4 hr	298 ± 9 (3)
24 "	328 ± 10 (10)
48 "	430 ± 12 (12)
7 days	432 ± 10 (5)

* All rats were sacrificed by neck clamp.
† Mean ± stand. error of mean (No. of rats).
All results expressed as mg adrenal ascorbic acid per 100 g fresh adrenal tissue.

no experimental treatment. Rats maintained for one week after cord-section exhibited normal adrenal ascorbic acid concentration.

Adrenal weight showed an increase after cord section. Adrenal weight of intact 250 g rats was 18.49 ± 0.8 (9) mg. Two days after cord section adrenal weight was 24.0 ± 0.8 (7) mg and at 7 days, 26.4 ± 0.8 (7) mg. *Effect of injected ACTH on adrenal ascorbic acid.* Dose response to injected ACTH was compared in 24-hour hypophysectomized and 2- and 7-day cord-sectioned rats. The members of the 3 groups had similar body weights. The rats were wrapped in a soft towel and the solutions in acid-saline (1 ml volume) were injected into a saphenous vein. One-half, 1.0 and 2.0 milliunits of U.S.P. ACTH Reference Standard, were employed (Table II). Response to ACTH was the same in the 3 groups. The sensitivity of the adrenals of 2- and 7-day cord-sectioned rats equals that of the 24-hour hypophysectomized rat.

TABLE II. ACTH Dose Response in 24-Hr Hypophysectomized Rats and in 2- and 7-Day Cord-sectioned Rats.

Exp. treatment	24-hr hypox rats	Cord-sectioned rats 2-day	7-day
Acid saline	434 ± 12* (12)†	430 ± 13 (9)	427 ± 17 (9)
.5 mU ACTH	384 ± 8 (16)	380 ± 10 (13)	392 ± 12 (8)
1.0 " "	356 ± 9 (13)	370 ± 13 (8)	379 ± 23 (8)
2.0 " "	314 ± 7 (13)	317 ± 10 (11)	325 ± 17 (8)

* Mean ± stand. error of mean.
† No. of rats.
All results expressed as mg adrenal ascorbic acid per 100 g fresh adrenal tissue.

Comparison of response to forepaw stimulation and hindpaw stimulation in cord-sectioned and in intact rats. Two important aspects of the electrical stimulation procedure were tested with intact rats: 1) the possibility that forepaw and hindpaw stimulation may induce different amounts of ACTH release, 2) the possibility that sham stimulation may elicit ACTH release in absence of electric current flow. The parameters of the electrical stimulus chosen resulted in moderate ACTH release in the intact rat. Application of this electrical stimulus to forepaws or to hindpaws of intact rats resulted in equivalent adrenal ascorbic acid depletion. Sham hindpaw or sham forepaw stimulation did not result in significant ascorbic acid depletion (Table III).

TABLE III. Adrenal Ascorbic Acid Concentration after Sham Stimulation or Electrical Stimulation of Forepaws or Hindpaws of Intact Rats.

Untreated	427 ± 16 (6)*
Sham forepaw stimulation	422 ± 10 (6)
Forepaw electrical stimulation	260 ± 18 (6)
Untreated	424 ± 23 (6)
Sham hindpaw stimulation	419 ± 20 (6)
Hindpaw electrical stimulation	254 ± 10 (6)

* Mean ± stand. error (No. of rats).
All rats sacrificed by neck clamp.
All results expressed as mg adrenal ascorbic acid per 100 g fresh adrenal tissue.

TABLE IV. Adrenal Ascorbic Acid Concentrations after Sham Stimulation or Electrical Stimulation of Forepaws or Hindpaws of 1-Week Spinal Cord-Sectioned Rats.

Untreated	432 ± 10 (5)*
Sham forepaw stimulation	404 ± 23 (6)
Forepaw electrical stimulation	310 ± 19 (5)
Untreated	444 ± 14 (8)
Sham hindpaw stimulation	435 ± 11 (9)
Hindpaw electrical stimulation	410 ± 18 (9)

* Mean ± stand. error (No. of rats).
All rats sacrificed by neck clamp.
All results expressed as mg adrenal ascorbic acid per 100 g fresh adrenal tissue.

The results obtained above in intact rats may be compared with results obtained in one-week cord-sectioned rats (Table IV). Ascorbic acid concentrations in untreated groups and in sham stimulated groups were similar to those obtained in intact rats. However, in contrast to the results obtained in electrically stimulated intact rats, only forepaw stimulation elicited significant adrenal ascorbic acid depletion in the cord-sectioned rat. Consequently intact spinal cord paths are essential for mediation of ACTH release following electrical stimulation of the periphery.

Discussion. The results indicate that intact neural paths in the spinal cord are essential for mediation of ACTH release following hindpaw electrical stimulation. No ACTH release was observed following hindpaw stimulation even though considerable directly and reflexly elicited muscular activity and elevation of blood pressure occurred during stimulation. This evidence supports the view that CRF is released by neural structures located in the brain plus cervical cord portion of the central nervous system, probably the hypothalamus, rather than from the periphery. These conclusions conflict with the interpretation of experimental results supplied by others(1,2,3,4,5). It is believed that this discrepancy is the result of methodological shortcomings and the failure of previous investigators to carry out sham control experiments to define the effective factor in stimulation procedures leading to ACTH release.

The results of indirect assays of blood ACTH levels by measuring activity of the adrenal of chronic preparations must be interpreted with caution. It is possible that altered adrenal metabolism under the peculiar conditions existing in certain chronic preparation may be misleading. Precautions have been taken in these experiments to avoid some of the common failures: 1) only spinal cord rats with adrenal ascorbic acid levels similar to unoperated controls were used, 2) responsiveness of the adrenals of the spinal rats was defined with a 3 point dose-response curve to U.S.P. Reference Standard ACTH, 3) capacity to release ACTH was established by demonstrating ascorbic acid depletion following stimulation above the level of cord transection.

After hindpaw stimulation in cord-sectioned rats adrenal ascorbic acid depletion was absent. After forepaw stimulation, ascorbic acid depletion occurred but was less than that observed in intact rats. The possibility was considered that the decreased depletion following forepaw shock was due to a reduction in adrenal sensitivity to ACTH in the cord-sectioned rats and that this reduction masked a small response to hind-paw stimulation. Sensitivity of adrenals of cord-sectioned rats to exogenous ACTH was investigated. Dose response to injected ACTH demonstrated that adrenals of 2- and 7-day cord sectioned rats responded similarly to adrenals of 24-hour hypophysectomized rats.

Other evidence obtained recently also supports the view that there is a depression in ACTH release in cord-sectioned rats which is independent of adrenal gland function. The difficulty of interpreting data obtained in chronic preparations in which altered adrenal sensitivity to ACTH may occur was eliminated by measuring blood ACTH levels in adrenalectomized rats. Furthermore, since the view is held that depression of ACTH release in cord-sectioned preparations is due to lack of a supporting secretion from the adrenal medulla, the adrenal medulla was eliminated. Under these conditions blood ACTH levels were depressed after cord-section in comparison to levels in control and sham operated adrenalectomized rats.

Summary. Spinal cord section results in early adrenal ascorbic acid depletion. Recovery of normal levels occurs after 48 hours and is maintained for at least one week. Cord-sectioned rats (2- or 7-day), exhibiting normal initial adrenal ascorbic acid levels, respond to injected ACTH with ascorbic acid depletion similar to that observed in 24-hour hypophysectomized rats. Adrenal ascorbic acid depletion response was employed as an index of blood ACTH levels. Intact rats respond to electric current flow through either forepaws or hindpaws with equivalent ACTH release. Cord-sectioned rats respond to forepaw stimulation with ACTH release, but do not respond to hindpaw stimulation. Sham stimulation of intact or cord-sectioned rats does not release ACTH.

Marion D. Lipscomb rendered valuable technical assistance.

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Pharmacologic Demonstration in Man of Increased Thresholds to Tooth Pain Following Carisoprodol and other Analgesics. (26166)

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Most methods for evoking experimental pain in humans trigger at the same time adjacent sensory fibers which interfere with precise perception and measurement of pain. Brashear(1) has observed that thermal, mechanical or chemical stimulation of a normal human tooth triggers pain only. No other sensation is experienced. Only pain fibers enter the pulp accompanying larger vessels and form an almost complete mantle around the arteries. The practicability of an algesimetric method based upon this neural structure is enhanced by constant conditions of stimulation afforded by a suitable electronic stimulator(2,3,4).

Methods. With a Burton "vitalometer,"* current was delivered to the base of the upper right incisor for 2 seconds, and increased by small increments at 5 to 10 second intervals until unequivocal recognition of pain was reported by the subject. One threshold measurement was made at each hourly trial. The contacting electrode was kept moist by frequently dipping it in 70% alcohol. Twenty normal men and women, 22 to 52 years of age, participated. Neither observer nor subjects had knowledge of the assignments of placebo or drug. One week elapsed between tests. On test days subjects reported to the laboratory at 9:00 a. m., when a control pain threshold record was obtained. Each subject then received 2 capsules of drug or placebo immediately after this test. Every subject received placebo 3 times during the series of trials. No foods or drinks were consumed until after all threshold tests had been completed. One hour after the drugs were ingested a second tooth pulp pain threshold was determined. A final threshold measurement was made 2 hours later (11:00). With initial readings (9:00 a. m.) as baseline, changes in tooth pulp threshold were calculated, and means computed for one-hour and 2-hour intervals. Biometric tests for significance of differences between groups are based upon "F" values derived by the analysis of variance(5).

Results. Carisoprodol at a single oral dose of 350 mg in gelatin capsules produced a statistically significant increase in pain threshold (130 ± 12.7% at one hour; 132 ± 12.4% at 2 hours) (Fig. 1). A single dose of 30 mg of codeine phosphate orally also raised pain threshold (128 ± 8.3% at one hour; 130 ± 10.1% at 2 hours). Placebo response did not differ significantly from baseline thresholds (96 ± 4.2% 104 ± 4.5%). Meprobamate in a single oral dosage of 800 mg failed to show any analgesic effect; the threshold value of 100 ± 7.4% did not differ significantly from placebo or baseline responses. In a single oral dose, acetylsalicylic acid at 1200 mg/person elevated pain threshold significantly at one hour (121 ± 14.5%). This response, however, was of relatively short duration since a statistically significant increase was not found at 2 hours. With 600 mg of acetylsalicylic acid, pain threshold was elevated (109 ± 9.2%), but not significantly. An oral dose

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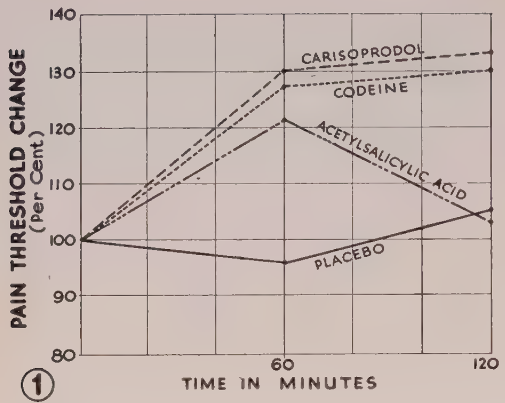
* Burton Mfg. Co., Santa Monica, Calif.



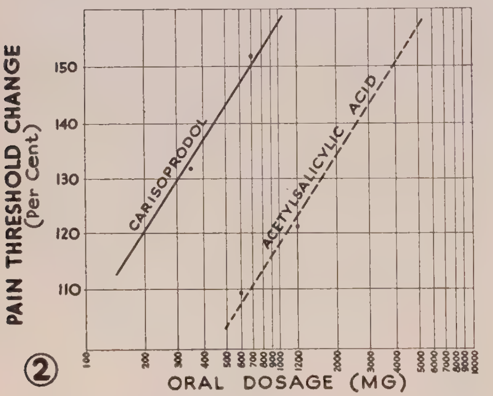


FIG. 1. % changes in tooth pulp threshold of humans following single oral doses carisoprodol (350 mg), codeine phosphate (30 mg), acetylsalicylic acid (1200 mg), or placebo. All preparations administered as capsules identical in appearance.

FIG. 2. Comparison of analgesia in man of carisoprodol and acetylsalicylic acid. Changes in tooth pulp threshold as a function of log-dose of drug.

of 700 mg of carisoprodol given as coated tablets markedly elevated pain threshold (151 ± 20.5% after 2 hours). A comparison of results obtained in this test suggests that carisoprodol was 5.4 ± 1.9 times more potent than acetylsalicylic acid in increasing tooth pain threshold (Fig. 2).

Discussion. Pharmacological studies in rats showed that there is a large ratio between doses of carisoprodol that produce paralysis and those causing insensibility to joint pain. This suggests that carisoprodol might possess significant analgesic properties in addition to being a potent skeletal muscle relaxant(6). Elevation of the tooth pain threshold following administration of carisoprodol was marked. Since the end-point did not require physiological intervention of muscle tissue for contraction, and was derived strictly from stimulation of pain fibers, it must be concluded that carisoprodol, like codeine phosphate or acetylsalicylic acid, acts as a true analgesic. Recently Holliday and Dille(7) described use of an ultrasonic stimulus for inducing deep bone pain in human volunteers. By this procedure, 700 mg carisoprodol orally resulted in a similar statistically significant rise in pain threshold. The intensity and duration of the pain threshold changes for acetylsalicylic acid follow changes in drug blood plasma concentration described by Mandel, *et al.*(8). The peak of response after this drug approximates maximal plasma concentration. The pharmacological response also follows very closely the clinical time-effect curves for acetylsalicylic acid reported by Houde, *et al.*(9). Likewise the response to carisoprodol follows closely the plasma level changes described for this drug. Maximal plasma concentration appears between 2 to 4 hours after oral administration(10).

Neuropharmacological studies by Kletzkin (11) and Longo(12) suggest the analgesic property of carisoprodol possibly derives from an action upon the reticular formation similar to that observed for morphine. Furthermore, the pharmacological demonstration in man of depressed sensitivity to pain involving the 2nd or 3rd divisions of the fifth cranial nerve suggests a clinical potential of carisoprodol for regional headache pain involving these divisions. Mephenesin in large intravenous doses has been reported to depress painful head sensations normally feeding through these divisions(13). Finally, a simple rapid means for assaying analgesic properties in man is afforded by the cited technic, and it seems to merit further trials with other analgesics. Since no significant analgesic action by the placebo can be shown by this method (Fig. 1), it affords a neurophysiological means of directly measuring analgesic action in the human.

Summary. 1. A pharmacological procedure is described which has clearly demonstrated the oral analgesic activity of carisoprodol (350 mg), acetylsalicylic acid (1200

mg), and codeine phosphate (30 mg) in man. Drugs were given with placebos in a "double-blind" investigation. Pain was induced by a high-frequency electronic stimulator applied to normal intact teeth. 2. Since the pain threshold end-point did not require activation of skeletal muscle, carisoprodol must have induced analgesia independently of its muscle relaxant action. 3. By this method, carisoprodol was 5 times more potent than acetylsalicylic acid in raising tooth pain threshold in man.

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Effect of Triethylene Thiophosphoramide (ThioTEPA) on Skin Pigmentation in Mice.* (26167)

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Hyperpigmentation of the skin of man and experimental animals following exposure to ultraviolet light or ionizing radiation is a commonly observed phenomenon. Radiation-induced hyperpigmentation has been shown to result from an increased amount of epidermal melanin associated with increased functional activity of the melanocytes in mouse(1) and human(2) skin. Similar results have been obtained in mice following local application of the carcinogenic hydrocarbon 5, 9, 10 - trimethyl - 1,2 - benzanthracene(3). During cancer chemotherapy studies in mice utilizing triethylene thiophosphoramide (ThioTEPA) it was observed that brown mice of the CBA and (RIII x CBA)F_1 hybrid strains undergoing long-term systemic administration of this drug developed a marked hyperpigmentation of the tail, ears, and extremities, similar to that described in mice exposed to chronic gamma-irradiation (1). The present study deals with the effects of ThioTEPA on skin pigmentation in various inbred strains of mice.

Methods and materials. ThioTEPA is the sulfur derivative of triethylene phosphoramide and is a member of the class of radiomimetic, tumor-inhibitory compounds known as alkylating agents. Dry crystalline ThioTEPA was dissolved in distilled water to make a concentration of 0.25 mg/ml and was refrigerated during the course of the experiment. Nine male and 9 female mice, 50 to 60 days old, of each of the following inbred strains were used:‡ DBA/2, Strong A, C3H, NH, Stoli, and (DBA/2 x NH)F_1 hybrids. Six males and 6 females of each strain were

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† Trainee, U.S.P.H.S. Cancer Training Grant.

‡ Obtained from Kirschbaum Memorial Research Lab., Dept. of Anatomy, Baylor Univ. College of Medicine.

TABLE I. Effects of ThioTEPA on Skin Pigmentation in Various Strains of Mice.

Strain	Coat color	Sex	Gross hyperpigmentation	Microscopic hyperpigmentation of skin	
				Ear	Abdominal wall
DBA/2	Dilute brown (non-agouti)	♂	—	Moderate	—
		♀	—	”	—
Strong A	Albino	♂	—	—	—
		♀	—	—	—
(DBA/2 × NH) F_1 hybrid	Black	♂	+	Marked	—
		♀	+	”	—
Stoli	Dilute brown (agouti)	♂	—	—	—
		♀	—	Moderate	—
C3H	Brown (agouti)	♂	+	Marked	—
		♀	+	”	—
NH	Piebald (gray & white)	♀	—	Moderate	—

randomly selected to receive ThioTEPA, the remainder serving as controls. The treated mice received 0.002 mg/g body weight of ThioTEPA (approximately 0.05 mg), injected intraperitoneally, daily for 5 days per week until a total of 12 injections had been given. Each mouse was observed during this 16-day period for grossly detectable pigment changes. On the day following last injection of ThioTEPA all the animals were killed and specimens of ears, gonads, adrenals, and skin over the anterior abdominal wall were obtained for histologic study. These tissues were fixed in 10% neutral buffered formalin, and paraffin sections 6μ thick were made following routine histologic procedures. Sections were either stained with hematoxylin and eosin or studied unstained.

Results. All males of the NH strain treated with ThioTEPA died during the course of this study. Pigment changes noted in the mice receiving ThioTEPA are summarized in Table I. In no case were any changes observed in the controls. Darkening of the tail, ears, and extremities of the ThioTEPA treated C3H and (DBA/2 x NH)F_1 hybrid mice occurred from 10 to 14 days after injections were begun, and was observed in all treated mice of these strains. Microscopic examination of the ears of these animals revealed an increased amount of melanin pigment incorporated within epidermal cells as well as an apparent increase in number of melanocytes in the basal layer of the epidermis (Fig. 1). The number of dermal melanocytes was definitely increased as compared to controls. No significant difference was detected in the melanocyte activity of hair follicles. None of the treated mice of the other strains exhibited gross hyperpigmentation, although a moderately increased amount of melanin pigment within the epidermis was noted in histologic sections taken from DBA/2 mice and females of the Stoli and NH strains. No gross or microscopic pigmentary changes were seen in the anterior abdominal wall skin of any of the mice in this experiment. Histologic examination of the adrenals failed to demonstrate any abnormalities. However, pathologic changes were seen in the gonads of almost all animals subjected to ThioTEPA. The testes of all treated male mice showed severe destruction of the seminiferous tubules with a sparing of interstitial cells. Alterations in the ovaries of treated females were less consistent, varying from no changes in the Strong A strain to almost complete obliteration of graafian follicles and absence of normal ova in the C3H strain.

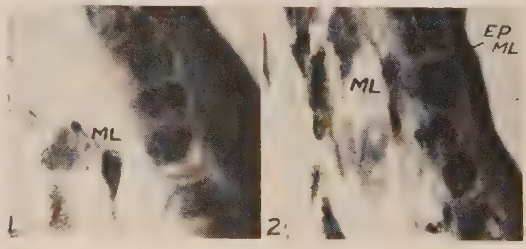

FIG. 1. Sections of skin from ears of (1) control and (2) ThioTEPA-treated C3H male mice showing melanocytes (ML) and melanin pigment deposited in cells of the epidermis (EP ML); × 507.

Discussion. ThioTEPA-induced hyperpigmentation of the extremities, tail, and ears of brown or black mice with sparing of the general body skin is similar to that described following total-body irradiation(1), both agents resulting in increased deposition of epidermal melanin and increased numbers of epidermal and dermal melanocytes. Presumably the greater number of melanotic melanocytes present in the extremities, tail, and ears(4) and the slower conversion of amelanotic to melanotic melanocytes in the general body skin(1) account for these findings. Szabó (5) has suggested that melanocytes of hair follicles can be stimulated with dimethylbenzanthracene and croton oil to function outside of their original environment. No significant increase in hair follicle melanocyte activity was detected in treated mice. The present study indicates, in addition, that similar microscopic changes occur to a lesser degree in mice of lighter coat color, but not in albinos. Further studies will determine whether ThioTEPA given in larger doses or for a longer time can cause gross hyperpigmentation even in these lighter coat-colored strains. The possible role of the adrenal or gonads in ThioTEPA-induced pigment changes is not clarified by these findings. All adrenals appeared normal histologically. All males and most females treated were apparently rendered sterile on the basis of histologic observations. It has been shown that ovariectomy will interfere with melanogenic activity(6).

Summary. Systemic administration of ThioTEPA caused the development of hyperpigmentation of the ears, tail, and extremities of brown or black mice, but not of mice of lighter coat color or albinos. These changes resulted from increased numbers of dermal and epidermal melanocytes and increased deposition of epidermal melanin. Similar microscopic changes were seen to a lesser degree in the lighter-colored mice, but not in albinos. Gross or microscopic evidence of hyperpigmentation of the general body skin was not found.

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Effect of Mycobacteria on Antibody Production Induced by Water-in-Oil Emulsions of Soluble Protein Antigens.* (26168)

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It has been well established that the sensitizing potency of tissue antigens in water-in-oil emulsions is augmented when mycobacteria are added. Thus, hypersensitivity states such as allergic encephalomyelitis, aspermatogenesis and thyroiditis are more readily produced when appropriate tissue antigens are incorporated into adjuvants containing mycobacteria than when the mycobacteria are omitted. On the other hand, the effect of mycobacteria on ability of antigens in emulsions to stimulate antibody formation is not clear. Addition of mycobacteria to water-in-oil emulsions containing viral, bacterial and rickettsial antigens has been reported to enhance greatly(1-3), to enhance slightly(4-5), or to have no enhancing effect(6-9) on production of circulating antibody. Also, numerous investigators have reported that mycobacteria or wax from mycobacteria greatly enhanced production of circulating antibody in animals injected with water-in-oil emulsions of soluble protein antigens(10-14), whereas others have reported that mycobacteria had little or no effect on antibody production in animals injected with emulsions of these same antigens(15-17). In preliminary studies in this laboratory with partially purified protein antigens, it was observed that mycobacteria enhanced production of circulating antibody in guinea pigs with one immunization scheme but not with another. The present investigation was initiated to determine the effect of mycobacteria on antibody production elicited by different immunization schemes.

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† This work was performed during the tenure as a Public Health Research Fellow of Nat. Inst. of Neurol. Dis. and Blindness.

Materials and methods. Protein antigens were obtained from the following sources: bovine gamma globulin (BGG), Armour and Co., Lot No. C-906 and C-904; and egg albumin (Ea), Armour and Co., Lot No. E-9015. BGG was trace labelled with I^{131} by the method previously described(18).

Adjuvants‡ were prepared with 9 parts bayol F (ESSO Standard Oil Co.), 1 part Arlacel C (Atlas Powder Co., Wilmington, Del.), and 10 parts of 0.15 M NaCl solution containing the protein antigen. The oil and emulsifier were first mixed in a homogenizer, then the protein solution was added and the mixture homogenized at 0-3°C until a paste-like emulsion was obtained. A similar adjuvant containing Arlacel A instead of Arlacel C was employed by Salk and Laurent(19). When mycobacteria (*M. tuberculosis hominis*) were incorporated into the adjuvant, they were mixed into the oil before addition of the emulsifier and protein solution. Male guinea pigs weighing 450-500 g were injected with a total of 15 mg of either BGG or Ea in adjuvant, with or without mycobacteria. Three different methods of injecting and bleeding were used. The first method was similar to that reported by Fischel *et al.*(12). Guinea pigs were injected once a week for 3 consecutive weeks with 1 ml of adjuvant containing 5 mg of antigen per ml. Half of the animals received adjuvant containing 0.64 mg of mycobacteria/ml, and half received adjuvant containing no mycobacteria. Each injection was given subcutaneously in multiple sites, including 0.1 ml in each foot pad. The guinea pigs were bled by cardiac puncture, 13 days, 4 weeks, and 7 weeks after last injection, and the sera were analyzed for precipitating antibody(20). The third method was similar to that described by White *et al.*(13). A single hind foot pad (subcutaneous) injection of 0.2 ml of adjuvant containing 15 mg of the antigen was given to each guinea pig. Half of the guinea pigs received adjuvant containing 1 mg mycobacteria and the other half received adjuvant containing no mycobacteria. The animals were bled 3 and 7 weeks after injection and the sera were analyzed for precipitating antibody. Guinea pigs were also injected in the same manner with adjuvant containing I*BGG with or without mycobacteria, and the disappearance of the radioactivity from both the whole animals and the foot pads was followed. The I* activity in both the entire animals and injection site (feet and lower hind legs) of these guinea pigs was determined periodically in a gamma well-type counter beginning on the first day following injection. Counts in the uninjected legs were less than 1% of the counts in the injected leg and were subtracted as non-specific background from the counts in the injected legs.

Results. High levels of precipitating antibody were produced in guinea pigs which received 3 weekly injections of BGG in adjuvant (3.0 ml total), both with and without mycobacteria (Table I). There was no significant difference in mean antibody concentration of the sera obtained from the 2 groups of animals during the period of study. However, 7 weeks after injection, the group receiving adjuvant without mycobacteria weighed 25% more than the group which received mycobacteria. Both those animals receiving mycobacteria and those not receiving

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‡ The term ''adjuvant'' will be used throughout this paper to denote a water-in-oil emulsion containing antigen.

TABLE I. Antibody Production in Guinea Pigs Receiving Multiple Injections of BGG in Adjuvant.

Guinea pig No.	μg antibody N/ml serum 13 days	4 wk	7 wk
(Without mycobacteria)			
1	532.0	592.5	—
2	768.0	—	—
3	652.0	—	—
4	506.0	684.5	729.5
5	493.0	796.0	748.0
6	642.0	695.0	722.0
7	681.0	—	—
8	507.0	734.5	635.5
9	599.0	977.5	836.5
10	296.0	420.5	—
11	960.0	927.0	792.0
12	407.0	418.0	312.0
13	820.0	844.5	771.5
14	569.0	771.0	710.0
15	535.0	—	—
Avg Ab. N/ml	597.8	714.6	695.2
Avg wt (g)	641	724	799
(With mycobacteria)			
1	473.0	597.0	567.5
2	258.0	273.0	—
3	297.0	603.5	524.5
4	439.0	629.5	608.5
5	741.5	696.0	—
6	725.0	—	—
7	706.0	582.0	—
8	690.5	683.5	—
9	745.0	770.0	774.0
10	568.0	724.0	718.0
11	559.0	834.5	741.0
12	377.5	595.0	580.5
13	613.5	752.0	749.0
14	404.0	578.0	—
15	604.0	—	—
Avg Ab. N/ml	546.7	639.8	657.9
Avg wt (g)	501	506	598

mycobacteria maintained high levels of antibody in their sera during the 7 week period. In fact, 7 weeks after last injection, there was more antibody present in the sera than 13 days following last injection. Similar results were obtained in guinea pigs which received 3 weekly injections of Ea in a total of 3 ml of adjuvant (Table II). In these animals, mycobacteria had a slight augmenting effect on concentration of precipitating antibody which was most noticeable soon after injections were terminated. However, the group of animals injected with mycobacteria did not gain weight as rapidly as did the group not injected with mycobacteria, so that total amount of antibody circulating per animal was somewhat greater in the animals receiving adjuvant without mycobacteria.

In contrast to the above results, mycobacteria had a marked enhancing effect on antibody production in guinea pigs given a single injection of BGG or Ea in 0.2 ml of adjuvant (Tables III and IV). Although the guinea pigs which did not receive mycobacteria gained somewhat more weight during the post injection period, this could account for only a small part of the difference in antibody levels.

A lesser augmenting effect of mycobacteria on antibody production was observed in guinea pigs which received a single injection of 3.0 ml of adjuvant (Tables V and VI). Antibody levels in sera of the group which received no mycobacteria were 35% (BGG) and 32% (Ea) lower at 7 weeks than those

TABLE II. Antibody Production in Guinea Pigs Receiving Multiple Injections of Egg Albumin in Adjuvant.

Guinea pig No.	μg antibody N/ml serum 13 days	4 wk	7 wk
(Without mycobacteria)			
1	82.2	81.6	74.6
2	341.2	376.0	402.2
3	182.2	259.6	296.4
4	270.4	—	—
5	128.4	116.8	109.2
6	192.2	240.0	222.4
7	381.6	470.0	457.2
8	240.4	267.2	364.0
9	350.0	356.4	362.4
10	401.6	442.4	464.8
11	385.6	—	—
12	175.2	163.6	233.2
13	302.0	326.0	—
14	87.6	103.8	111.6
Avg Ab. N/ml	251.5	266.7	281.5
Avg wt (g)*	625	689	764
(With mycobacteria)			
1	448.0	496.0	485.6
2	125.2	96.4	123.8
3	447.2	487.0	—
4	385.2	—	—
5	354.0	318.0	422.8
6	97.8	130.2	135.2
7	102.6	121.4	119.2
8	354.8	448.0	325.2
9	362.8	286.8	222.4
10	291.6	290.4	—
11	499.6	—	—
12	496.4	520.0	667.6
13	446.8	—	—
Avg Ab. N/ml	339.4	319.4	312.7
Avg wt (g)†	471	465	511

* Avg wt before inj. = 482.

† *Idem* = 496.

TABLE III. Antibody Production in Guinea Pigs Receiving a Single Injection of BGG in 0.2 ml of Adjuvant.

Guinea pig No.	μg antibody N/ml serum 3 wk	μg antibody N/ml serum 7 wk	Guinea pig No.	μg antibody N/ml serum 3 wk	μg antibody N/ml serum 7 wk
(Without mycobacteria)			(With mycobacteria)		
1	0	18.0	1	242.0	359.2
2	0	5.8	2	118.0	147.2
3	0	—	3	95.2	302.4
4	0	25.8	4	88.2	213.6
5	0	0	5	73.6	70.0
6	0	18.4	6	63.2	133.6
7	19.8	—	7	56.0	78.4
8	0	10.8	8	30.8	26.8
Avg Ab. N /ml serum		13.1		95.9	166.4
Avg wt, g*	545	708		481	606

* Wt at time of inj.: without mycobacteria, 472 g; with mycobacteria, 475 g.

in sera of the group which received mycobacteria. However, a large portion of this difference is offset by a difference in the weights of the animals. The groups which received no mycobacteria weighed 21% (BGG) and 22% (Ea) more at 7 weeks post injection than the groups receiving mycobacteria. Assuming a similar plasma protein pool to body weight ratio for both groups, there was only 21% (BGG) and 17% (Ea) less antibody produced in guinea pigs not receiving mycobacteria than in those receiving mycobacteria with this immunization scheme.

TABLE IV. Antibody Production in Guinea Pigs Receiving a Single Injection of Ea in 0.2 ml of Adjuvant.

Guinea pig No.	μg antibody N/ml serum 3 wk	μg antibody N/ml serum 7 wk	Guinea pig No.	μg antibody N/ml serum 3 wk	μg antibody N/ml serum 7 wk
(Without mycobacteria)			(With mycobacteria)		
1	102.4	49.6	1	124.0	
2	81.6	261.2	2	307.2	
3	7.6	20.4	3	349.6	244.0
4	104.4	202.8	4	398.4	324.2
5	74.0	180.8	5	372.6	—
6	26.6	—	6	416.4	—
7	29.2	52.0	7	391.6	430.4
8	54.2	—	8	255.2	362.0
9	24.2	40.6	9	359.2	—
10	15.4	47.0	10	342.4	369.6
11	10.8	11.8	11	147.6	224.0
Avg Ab. N /ml serum	48.2	96.2		306.3	325.7
Avg wt, g*	512	712		497	627

* Wt at time of inj.: without mycobacteria, 445 g; with mycobacteria, 447 g.

I*BGG in adjuvant injected into foot pads of guinea pigs disappeared somewhat more slowly when mycobacteria were incorporated into the adjuvant than when they were excluded (Table VII). A difference was not apparent until approximately 10 days after injection. The difference increased with time until, after 21 days, the injection sites in the

TABLE V. Antibody Production in Guinea Pigs Receiving a Single Injection of 15 mg BGG in 3.0 ml of Adjuvant.

Guinea pig No.	μg antibody N/ml serum 3 wk	μg antibody N/ml serum 7 wk	Guinea pig No.	μg antibody N/ml serum 3 wk	μg antibody N/ml serum 7 wk
(Without mycobacteria)			(With mycobacteria)		
1	57.2	212.8	11	335.2	468.6
2	254.4	320.4	12	135.2	232.8
3	207.6	182.8	13	426.6	374.8
4	150.8	242.4	14	341.6	—
5	63.4	101.6	15	147.6	208.8
6	231.2	224.4	16	396.6	—
7	226.8	—	17	345.4	472.2
8	267.6	—	18	362.6	333.6
9	56.4	104.8	19	422.2	—
10	63.0	364.8	20	427.0	358.0
Avg Ab. N /ml	157.8	219.3		334.0	349.8
Avg wt, g*	574			515	

* Wt at time of inj.: without mycobacteria, 461 g; with mycobacteria, 467 g.

TABLE VI. Antibody Production in Guinea Pigs Receiving a Single Injection of 15 mg Ea in 3.0 ml of Adjuvant.

Guinea pig No.	μg antibody N/ml serum 3 wk	μg antibody N/ml serum 7 wk	Guinea pig No.	μg antibody N/ml serum 3 wk	μg antibody N/ml serum 7 wk
(Without mycobacteria)			(With mycobacteria)		
21	197.4	249.8	31	556.8	546.7
22	286.2	220.2	32	324.0	—
23	153.4	187.4	33	556.8	487.1
24	105.7	—	34	592.4	515.6
25	277.2	—	35	323.6	462.0
26	357.6	509.2	36	600.8	534.4
27	534.4	531.2	37	638.0	—
29	312.8	299.2	38	214.2	361.6
30	199.8	292.2	39	266.6	—
			40	280.4	446.0
Avg Ab. N /ml	266.1	327.0		435.4	479.1
Avg wt, g*	588			490	

* Wt at time of inj.: without mycobacteria, 458 g; with mycobacteria, 465 g.

TABLE VII. Per Cent† of I*BGG Remaining in Injection Sites of Guinea Pigs.

Material inj.	No. of guinea pigs	Days following inj. 1	3	6	8	10	13	17	21	µg antibody N per ml serum 3 wk	7 wk
15 mg I*BGG in adjuvant											
Without mycobacteria	6	73.7	46.3	28.1	19.9	15.1	10.8	7.7	3.9	5.9	21.4
With mycobacteria	6	68.2	46.5	31.4	20.2	17.1	12.6	10.5	7.0	77.3	174.1

Guinea pigs were inj. in left hind foot pad with 0.2 ml of adjuvant.

† % of total I* activity inj.

guinea pigs which received mycobacteria possessed nearly 100% more I* activity than the group receiving no mycobacteria. At this time, the group which received the mycobacteria also had much higher levels of precipitating antibody. In another experiment in which I* activity was also determined in the whole body, a greater amount of I* activity was present after the tenth day in both the injection site and the whole body of guinea pigs receiving mycobacteria than in injection site and whole body of guinea pigs receiving no mycobacteria (Table VIII).

The granulomata developing around the depots of adjuvant with mycobacteria were consistently much larger than those developing about adjuvant without mycobacteria. This was true even when sites receiving only 0.2 ml adjuvant plus mycobacteria were compared with sites receiving 0.5 ml adjuvant without mycobacteria.

Initially, the histologic reactions to the adjuvant without mycobacteria, either in injection depot or in distant lymphatic tissues, were made up of a moderate number of lipophages, lymphocytes, a few plasma cells and occasional polymorphonuclear leukocytes. Within the next few weeks the number of lymphocytes and plasma cells about the lipid droplets increased and fibroblasts proliferated, in part walling off the droplets. During the first several weeks following injection, the number of large lipophages decreased. The granulomas that developed about the adjuvant with mycobacteria in the local depot or distant lymphoid tissues were conspicuous and consisted of great numbers of large mononuclear cells, some containing visible droplets of oil and others having a granular eosinophilic cytoplasm. Many giant cells were also present. These appeared to have been formed by coalescence of several large mononuclear cells. Moderate numbers of polymorphonuclear leukocytes, lymphocytes and plasma cells were also scattered through the granulomas. These granulomatous reactions were extensive, involving and frequently destroying adjacent striated muscle fibers. During the first several post injection weeks, some fibrosis took place about the droplets and occasional abscesses developed; otherwise the extensive granulomata remained histologically unchanged.

TABLE VIII. Per Cent of I*BGG Remaining in Guinea Pigs.

No. of guinea pigs	Area counted	Days following inj.† 3	5	7	8	10	14	16
	Without mycobacteria							
6	Foot	74	52	37	32	25	12	11
	Whole body‡	72	53	34	34	26	14	13
	With mycobacteria							
6	Foot	78	51	38	33	28	19	17
	Whole body‡	75	49	42	35	26	19	18

Guinea pigs were inj. in left hind foot pad with 0.2 ml of adjuvant containing 15 mg I*BGG.

† % of I* activity present on day 1.

‡ Minimal values, since efficiency of detecting isotopes throughout body is slightly less than detecting isotopes in localized area.

Discussion. The effect of mycobacteria in water-in-oil emulsions on production of precipitating antibody to a constant amount of protein antigen in guinea pigs depends on the immunization scheme employed. Mycobacteria had no significant augmenting effect on antibody production to either BGG or Ea when multiple injections totaling 3.0 ml of adjuvant were given over a 3 week period. When 3.0 ml of the adjuvant were given in a single injection to guinea pigs, 30-35% more antibody was obtained per ml of sera when mycobacteria were used than when they were not. However, when the antibody levels were calculated on a per animal basis, this difference was only 17-21%. On the other hand, mycobacteria had a marked enhancing effect on antibody production in guinea pigs injected with a single dose of 0.2 ml of adjuvant containing either BGG or Ea as previously demonstrated by White *et al.*(13). The amount of antibody produced following a single injection of 3.0 ml of adjuvant without mycobacteria was about the same as that produced after a single injection of 0.2 ml of adjuvant with mycobacteria. Since the same amount of antigen was given in both cases, it appears that in this instance a large volume of adjuvant lacking mycobacteria had as great an enhancing effect as did a small volume of adjuvant containing mycobacteria.

Perhaps the effectiveness of the antigen in adjuvant preparations determines the extent to which addition of mycobacteria will further enhance antibody production. In situations where the effectiveness in emulsion is great (3 ml of adjuvant in multiple injections), addition of mycobacteria to the adjuvant may not further enhance antibody production, but in situations where the effectiveness of the same amount of antigen is low (0.2 ml of adjuvant in a single injection), addition of mycobacteria may greatly enhance antibody production. The effectiveness of an antigen in an adjuvant preparation would be influenced by the immunization scheme employed, the species into which the adjuvant is injected, the antigen employed, and the physical state of the emulsion. Fischel *et al.*(12) observed a marked enhancing effect of mycobacteria on production of anti Ea in guinea pigs receiving multiple injections of Ea in adjuvant. The immunization scheme (amounts of antigen and mycobacteria injected, volume of adjuvant injected, etc.) was identical to one of the schemes (Tables I and II) used in the present study. The oil (paraffin) and emulsifying agent (aquaphor) differed from our oil and emulsifier and were mixed in different proportions. The adjuvant effect of the emulsion used by Fischel *et al.* was apparently inferior to that of the emulsion used in the present study, since the level of antibody produced in the present study without mycobacteria was approximately 3 times as high as that obtained by Fischel *et al.* with mycobacteria. Although all of the conflicting results published on the effect of mycobacteria on antibody production to antigen in adjuvant probably cannot be explained by a difference in the properties of the adjuvants employed, they may be explained by some other differences in the experimental situation which affect the effectiveness of antigen in adjuvant preparations; *e.g.*, animal species, volume of adjuvant injected, amount of antigen used, route of injection, or immunization scheme employed.

Adjuvants both with and without mycobacteria elicited a prolonged production of antibody. Antibody levels were generally higher 7 weeks following last injection than 2 to 4 weeks following last injection. A persistence of antibody production was observed in all of the animals in the present study and was independent of immunization scheme employed or level of antibody produced. This agrees with the work of Freund and Bonanto (21), who demonstrated agglutinating antibody in rabbits 3 years after injection of typhoid bacilli in adjuvant which either contained or lacked mycobacteria. On the other hand, Fischel *et al.*(12) reported that the precipitating anti Ea did not persist in sera of guinea pigs injected with adjuvant unless mycobacteria were added to the adjuvant. The physical state of the adjuvant employed might explain the failure of Fischel and coworkers to obtain prolonged antibody production without addition of mycobacteria.

There is no relationship between the size of granuloma produced at the injection site of adjuvant and antibody production. This is not surprising in view of the findings of White *et al.*(13), that only a small number of antibody containing cells in the local granuloma of guinea pigs injected with Ea in adjuvant either containing or lacking mycobacteria. Also, Askonas and White(22) demonstrated that the local granuloma produced by injection of adjuvant containing mycobacterium wax was relatively inactive in the *in vitro* synthesis of antibody. Apparently, mycobacteria do not serve to bring antibody producing cells to the local depot of adjuvant.

Our observations indicate a correlation between persistence of antigen and antibody formation, suggesting that one way in which mycobacteria might augment antibody response would be in increasing the retention of antigen. In the guinea pig, antigen was apparently eliminated more slowly from adjuvant with mycobacteria than from adjuvant without mycobacteria, and the former stimulated more antibody production. As might be expected, however, there is not a close correlation between amount of antigen retained and amount of antibody produced; mycobacteria and 0.2 ml of adjuvant elicited 12 times as much antibody in guinea pigs as did 0.2 ml of adjuvant alone, while mycobacteria and adjuvant only increased antigen retention by a factor of 2 over adjuvant alone. These data, while inconclusive, are consistent with the idea that mycobacteria in adjuvants enhance antibody production by increasing retention of the antigen.

Summary. In guinea pigs the effect of mycobacteria in adjuvant on production of precipitating antibody to a constant amount of antigen depends upon the immunization scheme employed. With multiple large injections of adjuvant, mycobacteria had no effect on antibody formation; with a single large injection, mycobacteria had a slight enhancing effect; and, with a single small injection, mycobacteria had an extreme enhancing effect. Mycobacteria had no effect on persistence of antibody production. There was a persistence of antibody production independent of the level of the antibody, with all the immunization schemes employed. Adjuvants containing mycobacteria produced considerably more extensive granulomata at injection sites than adjuvants lacking mycobacteria. However, there was no relationship between size of the granuloma produced at the injection site and antibody production. In guinea pigs injected with a small dose of adjuvant containing mycobacteria, there was both a greater persistence of antigen in the local adjuvant depot and in the whole body and a larger antibody production than when mycobacteria were omitted.

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Pharmacological Effects of Anthranilic Hydroxamic Acid and Certain Other Hydroxamic Acids. (26169)

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The pharmacology of hydroxamic acids has not been extensively studied. Epstein and Freeman(1) have determined the approximate LD_{50} in mice for a number of aromatic and aliphatic hydroxamates and their effectiveness against sarin poisoning. Askew(2) tested the toxicity of a number of other aromatic hydroxamates and found that most of them produced no overt symptoms at a dose level of 150 mg/kg. We have prepared hydroxamic acids some of which were toxic, but others were well tolerated at relatively enormous doses despite the fact that these compounds are able to chelate cations such as iron, magnesium and calcium. One compound, anthranilic hydroxamic acid, showed interesting pharmacological effects and is the main subject of this communication.

Experimental. The hydroxamates were synthesized from hydroxylamine and the methyl esters of the acids and isolated as sodium salts. The citric, succinic and malonic hydroxamates were very hygroscopic. They were dissolved in water and injected intraperitoneally into mice. One mg/g of the citric hydroxamate killed all of 10 mice in 5 minutes; half the dose caused no deaths. One mg/g of the succinic hydroxamate caused no deaths, but the mice were very depressed for several hours. Four mg/g of the malonic hydroxamate was tolerated without symptoms.

The sodium salt of anthranilic hydroxamic acid was recrystallized several times. It was dissolved in water and HCl added until the acid just began to precipitate at approximately pH 8.3. When it was injected intraperitoneally into rats, 1.0 mg/g, the animals lost consciousness in 10 minutes and remained anesthetized for 10 hours or more. Upon recovery they appeared normal and remained so. One half this dose caused depression for 8-10 hours but no loss of consciousness nor analgesia. Some excess salivation was apparent. Rats were trained for conditioned avoidance in a box with an electric grid for shocking and a fence to climb to avoid the shock. The conditioning shock was large enough so that for several days following the rats gave at least 10 consecutive positive reactions. When 0.5 mg/g of anthranilic hydroxamate was injected intraperitoneally, the reaction became negative after 10 minutes and remained so for the next 10 consecutive tests. The animals each time responded to the shock.

One half mg/g was given to rats every day. On the third day, the animals became somewhat restless before they were depressed. At this time they had lost no weight. But regularly on the fourth day they went into clonic convulsions, accompanied by excessive salivation, at intervals during 90 minutes. Depression then set in for about 8 hours. Injection on the fifth day caused more severe convulsions, which ended fatally. If injections were discontinued after the fourth day and not resumed until the eighth day, the rats behaved as they had originally. The magnesium and calcium salts had the same effect as the sodium salt, so the convulsions were not the result of chelation or depletion of these ions. Probably the drug accumulated in the body.

To test this, the urine of a rat was collected for 48 hours after it had received 1.0 mg/g of the anthranilic hydroxamate. $FeCl_3$ was added and the color read against a standard. Less than 10% of the dose appeared in the urine during this period. As a control an equivalent amount of malonic hydroxamate was given to a rat, and 83% of the dose was recovered in the urine after 24 hours. The anthranilic compound must either be stored or metabolized. Homogenates of rat liver or kidney were unable to metabolize it.

Anthranilic hydroxamate protected mice against twice the lethal dose of strychnine. 0.025 mg strychnine killed all of 5 mice within 6 minutes. Forty mg anthranilic hydroxamate given 10 minutes before the strychnine protected 7 out of 10 mice given 0.05 mg strychnine. Fifty mg and higher doses prolonged the life of mice given 0.1 mg strychnine, but they eventually died within a period of 90 minutes. The lives of mice given lethal doses of metrazol or picrotoxin were prolonged by anthranilic hydroxamate but there was no protection.

A dog was lightly anesthetized with nembutal. One g/kg of anthranilic hydroxamate was injected intravenously in a period of 30 minutes. During the next 2 hours, there were no changes in blood pressure, heart rate or EKG. The respiratory rate was slightly depressed at first, then became regular and shallow.

The hydroxamates of salicylic and benzoic acids in equivalent doses produced only transient depression, failed to abolish the conditioned avoidance response and could be given daily for 10 days without apparent ill effect as indicated by loss of body weight. They showed no protection against strychnine, metrazol or picrotoxin. The hydroxamate of phthallic acid is toxic; 0.25 mg/g is the lethal dose. When half this dose was given, tremors occurred at intervals for several hours, and the rat appeared very sensitive to touch. Respiration was of the Cheyne-Stokes type. Anthranilic hydroxamate antagonized these effects.

Discussion. Salicylic and anthranilic hydroxamates should be almost equally effective as chelating agents. Since their pharmacological actions are so different, chelation probably does not play an important part in the mechanism of action of anthranilic hydroxamate. This compound appears to penetrate the blood-brain barrier rapidly and the data suggest that its action is central. Despite the salivation, the absence of effect on blood pressure and heart rate indicate that the autonomic system is not affected centrally or peripherally. It will be of interest to study its effect on brain metabolism.

Summary. The hydroxamate of anthranilic acid injected intraperitoneally into rats in a dose of 1.0 mg/g causes anesthesia lasting 10 hours or more. Recovery is apparently complete. One half this dose causes tranquilization and suppression of the conditioned avoidance response. The compound protects mice against twice the lethal dose of strychnine. It merely prolongs the life of animals given metrazol or picrotoxin. A dose of 1.0 g/kg injected intravenously in a dog has no effect on heart rate, EKG or blood pressure and only minor effects on respiration. Equivalent amounts of the hydroxamates of salicylic and benzoic acids have none of the pharmacological effects.

Our thanks are due to Mr. W. J. Novick and Dr. E. M. Scarpelli for assistance in this study.

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Effect of Arsenic on Growth of Mammalian Cells *in vitro*.* (26170)

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Arsenicals are useful in veterinary medicine (1,2), and in the past decade they have also acquired importance in animal nutrition as growth stimulants(3). Interest in the metabolic turnover of the growth promoting arsenicals and the question of tissue residues has spurred the need for additional basic information on the physiologic role of this class of compounds. The application of tissue culture methods to determine the direct response of mammalian cells to a variety of arsenicals was the immediate aim of the present study.

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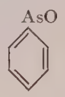
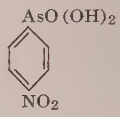
Through the standard technics of tissue culture based on the classical tissue explant-plasma clot preparation, arsenic has been reported to exert both growth promoting(4,5,6) and toxic(7,8,9) effects. Subsequent advances in tissue culture have made it possible to grow large numbers of homogeneous cells descended from a common ancestor,—a single isolated cell. Cultivating tissue cells dispersed in readily prepared fluid media of reproducible composition has overcome some of the limitations of the plasma clot and has improved the quantitative aspects of cell culture. In the present work the effect of arsenicals in the immediate chemical environment of living cells was ascertained quantitatively by measuring survival and proliferation of human and animal cells in replicate glass monolayer cultures.

Methods. The growth response of 4 strains of mammalian cells was determined after a period of continuous exposure to various arsenicals. Solutions of the arsenicals, sterilized by filtration through fritted glass, were added to suspensions of the cells in growth medium composed of 90% Eagle's basal medium[‡] and 10% horse serum. The medium also contained penicillin (100 units/ml), streptomycin (100 mmg/ml), tetracycline (10 mmg/ml), and nystatin (50 units/ml). There were 4 to 6 replicate cultures for each dose of arsenical. The cell strains were human skin of substrain 2198(10), HeLa S-3, Chang's human liver, and Earle's L-929 mouse fibroblast. Cells were grown for 4 days in stationary cultures containing 2 ml of nutrient in T-9 flasks(11), 10 flasks to a rack,[§] and incubated at 37°. At the end of 4 days the cells were harvested by trypsinization, washed with saline, acid hydrolyzed, and assayed for deoxyribonucleic acid (DNA), which is synthesized in the cell nuclei.

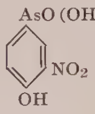
In addition to sodium arsenite and sodium arsenate, 4 organic arsenicals used as feed supplements(12) were also tested. One of these was arsenosobenzene, a potent coccidiostat containing trivalent arsenic. The remaining compounds were derivatives of phenyl arsonic acid (As^V), *viz.*, 4-nitrophenyl arsonic acid (4NA), 3-nitro-4-hydroxyphenyl arsonic acid (3-4-A), and arsanilic acid.



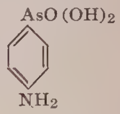
arsenosobenzene
(phenyl arsenoxide)



4-nitrophenyl
arsonic acid (4NA)



3-nitro-4-hydroxyphenyl
arsonic acid (3-4-A)



arsanilic acid

Since the low pH of solutions of 3-4-A acidified the culture medium, this compound was neutralized before filter sterilization. Arsanilic acid was tested as the sodium salt (atoxyl). Arsenosobenzene was brought into solution by first neutralizing with a drop of alkali followed by several hours of magnetic stirring. Studies of sodium arsenite and arsenate were carried out simultaneously, the same controls serving both series.

Throughout the present studies, measurement of growth response was based on the demonstration that DNA serves as a reliable index of size of cell populations cultivated either in natural or synthetic media(13). The DNA content of a culture gives a relative estimate of total cell number and avoids the problem of obtaining an accurate count of degenerating cells in aliquots of suspensions from cultures with high growth inhibition or mortality. The procedure selected for assaying DNA was an adaptation of a quantitative colorimetric test for deoxyribose(14). This method required some modification for application to tissue culture, and the variations are therefore described briefly.

DNA Assay. The contents (2 ml) of each T-9 culture flask are transferred to a 15-ml conical centrifuge tube. The flasks are rinsed with saline after removing the adherent cell layer with 0.125% trypsin. The harvested cells are centrifuged down, and supernatant is discarded. The cells are then resuspended in saline. The centrifuging is repeated, following which the saline is replaced with 3 ml of 5% trichloroacetic acid (TCA). The cells are hydrolyzed with TCA for 30 minutes in a gently boiling water bath while the centrifuge tubes are loosely stoppered with sealed ampoules. After cooling in water, 1 ml more of TCA solution is added to each tube and the acid-insoluble precipitate is centrifuged down. The hydrolysate is transferred to a 15-ml graduated, glass-stoppered, centrifuge tube, and the volume is adjusted to 4.0 ml. The unknowns, reference solutions containing from 10 to 100 mmg DNA‖ in 4 ml of TCA, and a blank consisting of 4 ml of 5% TCA, each receive 0.2 ml of a 0.4% alcoholic solution of p-nitrophenylhydrazine. With sealed ampoules as condensers, the tubes are heated for 20 minutes in a gently boiling water bath. After cooling, 9 ml of *n*-butyl acetate are added to each tube, glass stoppers are applied and the tubes well shaken. The stoppers are removed, the tubes centrifuged and the upper layer aspirated off. With finger in place over the end of the pipet as the tip passes through the organic phase, 3 ml of the lower (aqueous) phase are pipetted into a 5-ml volumetric flask, and 1 ml of 2*N* NaOH is added for color development. The solution is made up to volume with water, mixed, and read in a colorimeter at 560 mμ. A Spectronic 20 has been found suitable for this purpose.

[‡] Microbiological Assoc., Inc., Bethesda, Md.

[§] Kontes Glass Co., Vineland, N. J.

Substances interfering at 560 mμ, such as the serum and the phenol red of the basal medium, were absent in the second supernatant when checked with the Beckman DK-1 spectrophotometer. Excesses of p-nitrophenylhydrazine causing occasional colorimetric errors are averted by preparing the dye as a 0.4 instead of the 0.5% solution originally prescribed(14). The use of glass-stoppered centrifuge tubes eliminates the possibility of color extraction from rubber stoppers by butyl acetate. The standard error of a representative series of 5 control cultures with a mean DNA content of 47.7 mmg (Table I, arsonic acid) was ± 0.8 mmg of DNA.

TABLE I. Inhibition of Mammalian Cell Growth by Arsenicals as Measured by DNA Content of Cell Cultures.

Arsenosobenzene (phenyl arsenoxide) Cell strain L-929			3-NO_2-4-OH-phenyl arsonic acid (3-4-A) Human skin, substrain 2198		
As^{III} (ppm)	DNA content (mmg)	Growth inhibition (%)	As^{V} (ppm)	DNA content (mmg)	Growth inhibition (%)
.00 *	17.1		0*	20.7	
.00 †	53.8	.0	0†	47.7	.0
.005	52.0	4.9	10	44.8	10.7
.015	47.6	16.9	30	42.2	20.4
.025	38.6	41.4	50	39.2	31.5
.035	30.7	62.9	100	32.5	56.3
.05	17.0	100.0	300	24.4	89.6
			500	22.0	95.2

* 0-day controls (inoculum).
† 4-day control cultures.

Results and discussion. The magnitude and range of variation in DNA over the 4-day test period in 2 typical experiments are indicated in Table I. The difference in magnitude of the concentration range of these 2 arsenicals is reflected in the difference in slopes of the respective dose-response curves. A plot of percent growth inhibition of skin cells by 3-4-A *vs.* logarithm of arsenic concentration gives a relatively gradual slope, or increase in growth inhibition with progressive increase in concentration of arsenic. Concentrations of arsenic corresponding to a 50% suppression of cellular proliferation (Table II) were derived from either log-probability(15) or semilogarithmic plots,—the respective values being almost identical in most instances. The magnitude of the plotted slopes bore no consistent relation either to valence of arsenic or to cell strain.

The reaction of all 4 strains of tissue cells to any single arsenical was fairly similar. All cells were decidedly most sensitive to arsenosobenzene, more so than to inorganic arsenite (Table II). Arsenosobenzene is the nitrogen-free, reduced prototype of the arsonic acids. A possible mechanism for the strong lethal action of arsenosobenzene may lie primarily in the greater permeability of the cell wall to this compound, following which arsenosobenzene may block more enzyme systems than

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‖ California Corp. for Biochemical Research, Los Angeles, Calif.

TABLE II. Concentration of Arsenic Effecting 50% Growth Inhibition of Mammalian Cells.

Compound		Cell strain L-929	Skin	HeLa	Liver
Arsenosobenzene	(As^{III})	.029	.029	.044	.022
Sodium arsenite	(As^{III})	.19	.22	.13	.14
" arsenate	(As^{V})	1.6	4.7	2.0	4.0
4-NO_2-Ø arsonic acid	(As^{V})	10.3	28.6	26.1	18.7
3-NO_2-4-OH-Ø arsonic acid	(As^{V})	46.2	84.	92.	44.7
Sodium arsanilate (atoxyl)	(As^{V})	73.9	122.	64.8	117.

Values are expressed in parts/million of elementary arsenic.

do other arsenicals. Eagle and Doak(16) have reviewed the permeability theory, but did not attempt to identify the factors determining which compounds can penetrate the cell wall and which cannot.

Trivalent inorganic arsenic was as inhibitory at 0.1 to 0.2 ppm as was the pentavalent form at 2 to 5 ppm. At least 5 to 10 times as much organic pentavalent arsenic (4NA) was required to effect the degree of growth suppression produced by the element in the form of the arsenate. Skin and liver cells showed the widest range of sensitivity to the arsenicals. To suppress growth 50%, all cells required from 1500 to 5000 times as much arsenic in the form of atoxyl as in the form of arsenosobenzene. It is interesting that 3-4-A was exceptionally well tolerated by HeLa cells. At 300 to 500 ppm of 3-4-A growth of HeLa cells was suppressed only 60%. Results with cultured mammalian cells in many respects parallel the findings with *Lactobacillus leichmannii* reported from this laboratory (17). The microbiological studies likewise demonstrated higher growth suppression by the trivalent arsenicals and showed arsanilic acid to be least inhibitory of the compounds tested.

Arsenic content of the medium and detoxifying ability of the serum were not determined. It is possible that a formulation of some protein-free medium may demonstrate a growth stimulating influence of a specific arsenical on a particular pure cell line. In one of our preliminary trials a growth increment 50% above that of control skin cultures was noted in the presence of arsenate (1.5 ppm As^{V}). This observation was not confirmed by subsequent tests. The arsenate may have substituted for or replaced phosphate in some enzymatic reactions and increased enzyme activity, oxidation, or overall rate of reaction. The subject of arsenolysis is outlined in a recent review of the biochemistry of arsenic(2).

Wide variation in the potency of arsenicals *in vivo* from a pharmacologic standpoint is generally considered to be related to the difference in valence of arsenic. This variation was emphasized by the response of rapidly multiplying mammalian or bacterial cells *in vitro*. Both types of cell reacted to the inorganic and organic trivalent compounds by a reduction of cellular proliferation exceeding that produced by the pentavalent arsenicals. From a nutritional and metabolic standpoint it would appear, therefore, that throughout the course of absorption, distribution, uptake, retention, and elimination of arsenicals in the animal body the arsenic content of the compounds may be of far less consequence than the valence of the element.

Summary. Under continuous exposure to arsenicals *in vitro*, mammalian cells showed a remarkably wide range of tolerance to arsenic. To suppress growth of cells by 50% the absolute quantity of arsenic administered as atoxyl was up to 5000 times that in the form of arsenosobenzene. Pentavalent organic arsenicals with nitrogen groups were comparatively well tolerated in cell culture. In cells originating from different tissues and cultured in the same medium, degree of growth inhibition by a particular arsenical was fairly uniform. An adaptation of a DNA assay, useful in estimating cell populations in tissue culture, is described.

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A Study of the Relationship Between Canine Distemper and Measles in the Dog.* (26171)

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Evidence has been presented for(1-3) and against(4,5) the suggestion that the viruses of human measles and canine distemper are antigenically related. This report describes the results of inoculation of dogs with live measles virus and subsequent challenge with virulent canine distemper virus. Despite the minimal canine distemper antibody response which followed the measles inoculation, all these dogs resisted challenge with distemper virus.

Materials and methods. The Edmonston strain of measles virus(6) was obtained from Dr. John F. Enders, after 28 transfers in human kidney tissue culture. Two pools of live measles virus were used. Pool 1 had been passaged 3 times in rhesus monkey kidney tissue culture and had an infectivity titer of $10^{4.0}$/0.1 ml in that system. Pool 2 was in the 6th tissue culture passage and titered $10^{4.5}$/0.1 ml. Control material consisted of uninoculated monkey kidney tissue culture fluid derived from the same batch of cells in which the measles virus was produced.

The Snyder Hill strain of distemper virus, maintained by serial intracerebral transfer in dogs, was previously reported(7) to produce convulsions and/or death in dogs with regularity, when given intracerebrally. This same strain, when administered as spleen suspension by the intravenous route, produced the classical disease described by Dunkin and Laidlaw(8).

The dogs used in these studies came from a purebred colony of beagles, reared and maintained in isolation by the Veterinary Virus Research Inst. The 19 dogs represented 6 different litters, ranging in age from 5 to 24 weeks at time of measles injection. Each litter was placed together in a separate isolation unit for observation a few days prior to inoculation.

Three groups of dogs were inoculated with measles virus or control tissue culture fluid, then challenged with distemper virus, the time and route of challenge varying with each group. Group A (Tables I and II) included 9 dogs, consisting of 3 separate experiments with litters of 3 dogs each. In Group A, 2 dogs of each litter were injected intravenously

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TABLE I. Protective Effect of Live Measles Virus Inoculation against Subsequent Canine Distemper Virus Challenge in Dogs.

				Distemper challenge		Effects of challenge		
Group	No. of dogs	Inoculation material	Clinical response	Time (days)	Route	Fever	Convulsions	Died
A	6	Measles	None	24–28	Intrav.	0/6*	0/6	0/6
	3	Tissue culture control	"	24–28	"	3/3	0/3	1/3
B	2	Measles	"	84	"	0/2	0/2	0/2
	2	Tissue culture control	"	84	"	2/2	0/2	0/2
C	3	Measles	"	45	Intracer.	2/3†	0/3	0/3
	3	Tissue culture control	"	45	"	3/3	3/3	2/3

* Fraction = No. positive/No. tested. † Fever lasted only 2 days.

with 1 ml of live measles virus. The remaining dog was injected intravenously with 1 ml of control tissue culture fluid. Twenty-four to 28 days after injection, each dog was injected intravenously with 1 ml of a 10% suspension of spleen containing Snyder Hill virus strain. Group B consisted of 2 dogs inoculated with measles virus and 2 with tissue culture control material. The distemper virus challenge was delayed until 84 days after inoculation. In Group C, 2 litters of 4 and 2, respectively, were used as split litters kept together in the same unit. Three dogs were injected intravenously with 1 ml of measles virus. The other 3 were injected intravenously with 1 ml of control tissue culture fluid. Forty-five days later, each dog was injected intracerebrally with 0.25 ml of a 20% brain suspension of Snyder Hill virus.

In all experiments, the dogs inoculated with tissue culture control material were maintained in the same isolation unit as their litter mates inoculated with measles virus. Dogs in Groups A and B were given measles virus pool 1, and those in Group C were given pool 2. Serum was obtained from the dogs prior to each injection and 28 days after challenge with distemper virus, if still living. Temperatures were recorded and animals observed for signs of illness daily. The abdomen was shaved on some of the dogs, so that a rash could be detected more readily.

The egg-adapted Onderstepoort strain of distemper virus was used in the distemper neutralization test. After inactivation at 56°C for 15 minutes, each serum was diluted in phosphate buffered saline, pH 7.2. Each serum dilution was mixed with an equal volume of virus that contained 300 EID_{50} of Onderstepoort virus. The mixtures were incubated at 6°C for 2 hours. A group of 5 fertile eggs that had been incubated for 7-8 days was inoculated with 0.2 ml of the mixture on the chorioallantoic membrane, by the method of Gorham(9). The eggs were examined for lesions after further incubation of 6-7 days.

In the measles neutralization test, approximately 100 $TCID_{50}$ of measles virus was incubated with serum dilutions at room temperature for 1 hour. The mixture was inoculated into monkey kidney tissue culture and the endpoint read after 7 days' incubation. Measles complement-fixation tests were performed using untreated virus grown in monkey kidney tissue culture as antigen.

Results. All dogs given measles virus intravenously remained well and developed no rash. Twenty-four to 28 days after measles injection, the measles neutralizing titers of 6 dogs in Group A ranged from 64 to greater than 256 (Table II). Despite the substantial measles neutralizing antibody response, there was a minimal distemper response. The distemper neutralizing titers of 4 dogs were less than 2.5 and the other 2 had titers of 5 and 7, respectively. The measles neutralizing titers of 2 dogs in Group B, 84 days after measles injection, were 64 and 128, while distemper titers were 5 and 12. Measles comple-

TABLE II. Neutralization Response of Dogs to Measles Virus and Canine Distemper Virus.

			Neutralization titers*					
			Measles			Canine distemper		
Group	Inoculation material	Dog No.	Pre-measles inoc.	Pre-distemper challenge	Post-challenge	Pre-measles inoc.	Pre-distemper challenge	Post-challenge
A	Measles	753	<4	128	32	<2.5	<2.5	1,100
		754	<4	128	1024	<2.5	<2.5	10,000
		728	<4	>256	256	<2.5	<2.5	500
		731	<4	64	64	<2.5	<2.5	400
		771	<4	128	128	<2.5	7	300,000
		772	<4	128	128	<2.5	5	8,000
	Tissue culture control	752	<4	<4	trace	<2.5	<2.5	55
		729	<4	<4	<4	<2.5	<2.5	15,000
		770	<4	<4	died	<2.5	<2.5	died
B	Measles	768	<4	128	>1024	<2.5	12	300,000
		769	<4	64	256	<2.5	5	4,000
	Tissue culture control	766	<4	<4	<4	<2.5	<2.5	30,000
		767	<4	<4	<4	<2.5	<2.5	300,000
C	Measles	786	<4	32	128	<2.5	28	3,000,000
		790	<4	N.D.	256	<2.5	28	300,000
		787	<4	128	32	<2.5	30	60
	Tissue culture control	788	<4	<4	died	<2.5	<2.5	died
		789	<4	<4	<4	<2.5	<2.5	100,000
		791	<4	N.D.	died	<2.5	<2.5	died

* Reciprocal of 50% serum neutralization endpoint. N.D. = not done.

ment-fixing antibody rise paralleled the neutralizing response in all cases. The 5 unit contact dogs in Groups A and B, given control tissue culture fluid, remained well. They had no demonstrable measles or distemper antibodies after 24 or 84 days.

When given Snyder Hill virus intravenously 24-84 days later, the 8 dogs inoculated with measles virus (Groups A and B) showed no signs of illness. Twenty-eight days later, their measles antibody titers ranged from 32 to greater than 1024, and distemper antibody titers ranged from 400 to 300,000. The 5 unit control dogs that were similarly injected showed typical signs of distemper infection, with a diphasic temperature curve and catarrhal signs, and 1 died. The 4 survivors had distemper antibody titers ranging from 55 to 300,000 twenty-eight days later. Dog No. 752 with the titer of 55 had a more severe clinical illness than the other survivors. Two months after challenge, when complete recovery had taken place, its serum titer was 2250. Injection of Snyder Hill virus into the unit control dogs produced no measles neutralizing antibodies, or only a trace.

The Group C dogs given measles virus developed measles neutralizing antibody titers of 32 and 128 and distemper neutralizing antibody titers of 28 to 30, on the 45th day. Following intracerebral challenge with Snyder Hill strain, 1 dog (No. 787) showed no signs of illness, whereas the other 2 dogs (Nos. 786 and 790) had a definite febrile reaction 2 days after inoculation, for 2 consecutive days, with no other observable signs. These dogs developed distemper antibody very rapidly and to high titer. Eight days after intracerebral inoculation, the distemper neutralizing antibody titers were 25,000 and 250,000, and at 28 days, 300,000 and 3,000,000, respectively. In contrast, the non-febrile dog had a neutralizing titer of 60 at the 8th and 28th days. The 3 Group C unit contact dogs had no neutralizing antibodies to distemper virus or measles virus at the time of intracerebral distemper challenge. These dogs developed characteristic central nervous system manifestations and 2 died at 7 and 8 days post-inoculation. The survivor had a distemper neutralizing titer of 7 at 8 days and 100,000 at 28 days after challenge.

Discussion. The challenge tests demonstrate that live measles virus given intravenously protects dogs against virulent distemper virus given 24-84 days later. Control dogs failed to develop neutralizing and complement-fixing antibody to measles virus and were presumably not infected, though they were maintained in close contact with dogs inoculated with measles virus. Furthermore, they became ill when challenged with distemper virus.

Our data support the concept that these 2 viruses have an antigenic relationship. Of the 11 dogs inoculated with measles virus, 7 developed low but definite distemper neutralizing titers, ranging from 5 to 30. However, the possibility that interference may be operative in the protection afforded by measles virus is difficult to eliminate entirely. The dependence of interference upon continued association of the interfering agent with the host cell(10) makes this unlikely, in view of the long period (84 days) over which the protection was demonstrable. The antibody response to live measles virus in the dog suggests that the virus replicates in that host. Following a single intravenous inoculation of measles virus, high titers of both neutralizing and complement-fixing antibodies are produced in the dog, quantitatively resembling the response to measles infection in man or monkey. It has been reported(11) that measles virus was propagated serially in puppies, producing mild illness with viremia, fever and rash. For these studies, virus obtained directly from blood and pharyngeal washings from children in the acute stage of measles was used, as compared to the tissue culture adapted Edmonston strain used here.

Studies by Warren *et al.*(3) agree closely with the present experiments. These authors demonstrated production of measles neutralizing and complement-fixing antibodies in dogs, following inoculation with live measles virus grown in human heart tissue culture. Despite the fact that relatively low levels of canine distemper virus neutralizing antibody resulted from measles immunization, a partial protection against intraperitoneal challenge with virulent Snyder Hill strain of distemper virus was observed by these authors. On the other hand, it was found that the ferret was resistant to infection by measles virus, as judged by failure to produce measles complement-fixing antibody in that host. Furthermore, inoculation of ferrets with live measles virus did not protect against distemper challenge. Cabasso *et al.*(5) similarly failed to protect ferrets against distemper by hyperimmunization with measles virus-adjuvant mixture.

The rapid and high titered canine distemper neutralizing antibody response following intracerebral challenge in 2 dogs of Group C (Nos. 786 and 790) may be interpreted as an anamnestic response. The dogs had brief febrile responses and developed titers of 25,000 and 250,000 on the 8th day following canine distemper virus administration. Such levels are usually not attained following primary infection until 3 to 4 weeks have elapsed. This brief febrile response and anamnestic reaction has been observed recently in distemper immunity studies in dogs given a series of 2 or 3 injections of inactivated distemper virus at two week intervals, followed by intravenous injection of virulent distemper virus 3 months later. The third dog in Group C (No. 787) had no illness and no increase in titer, suggesting that sufficient protection was present at time of intracerebral inoculation to interfere with production of illness and antibody increase. Similar results have been observed in studies of the immune mechanism in distemper(12,13), *i.e.,* a low antibody titer (20-80) may in some individuals result in an apparent complete resistance to distemper virus replication, and consequently no illness or increase in neutralizing antibody titer occurs.

These studies strengthen the case for an antigenic relationship and emphasize the need for virus challenge as well as serologic studies in establishing the immunological relationship of viruses.

Summary. Dogs inoculated with live measles virus readily developed neutralizing and complement-fixing antibodies to measles. The dogs showed no signs of illness and did not spread the infection to unit contact litter mates. Following measles inoculation, 7 of 11 dogs developed low titers of canine distemper neutralizing antibody. All measles-inoculated dogs, including those with no demonstrable canine distemper neutralizing an-

tibody, were protected against intravenous or intracerebral challenge with virulent canine distemper virus, while the contact controls, not receiving measles virus, became ill, and some died. The dogs which had received distemper virus alone developed distemper neutralizing antibody, but developed no measles neutralizing antibody.

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The Effect of Niacinamide on Cerebral Circulation. (26172)

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It has been reported previously(1,2) that niacinamide has no significant effect on cerebral blood flow when given in doses of 50 to 100 mg intravenously. Large doses of the drug (3-5 g, i.v.) have been shown to increase cardiac output in the dog to a remarkable degree(3) and it was thought that there might be an associated increase in cerebral blood flow. Consequently the effects of small and large doses of niacinamide on cerebral circulation were determined to test this hypothesis.

Method. Dogs were anesthetized by pentobarbital 40 mg/kg i.v. Cerebral blood flow was measured by the N_2O method described by Kety and Schmidt(4-6). The sagittal sinus was exposed and punctured by a needle (15 gauge) and connected by polyethylene tubing with a 3-way stopcock, a manometer (1 mm I.D., 45 cm height) and a heparinized sampling syringe(6,7).

As we had no ready made N_2O(15%)-O_2(21%)-N_2(64%) mixture we mixed O_2 and N_2O in the ratio of 60 to 40 by a flowmeter. The O_2 content of the mixture was analyzed by a Scholander gas analyzer(8) and it was found that the gases mixed successfully in the desired ratio.

Control blood samples were obtained 15 min. after puncture of the sinus. After another 15 min interval, the drug was injected intravenously in 20 ml of water and 5 to 15 min later blood samples were taken again. The samples were analyzed in a Van Slyke-Neill manometric apparatus by the method of Orcutt and Waters(9). Mean arterial blood pressure (MABP) readings were taken from the femoral artery. O_2 consumption of the brain ($CMRO_2$) was determined by multiplying arteriovenous O_2 difference and cerebral blood flow (CBF). The data were treated by t-test(10).

Results. The effect of large doses of niacinamide (350 mg/kg) on cerebral circulation in 7 dogs 5-15 min after injection is shown in Table I. CBF/100 g/min increased significantly from 39.4 ± 7.2 (mean ± s.e.) to 56.2 ± 12.0 ml ($P<.01$), and $CMRO_2$/100 g/min. from 2.71 ± 0.85 to 4.18 ± 1.47 ml ($P<.05$), while MABP decreased from 139.3 ± 22.3 to 95.0 ± 16.6 mm Hg ($P<.001$)

TABLE I. Effect of Large Doses of Niacinamide on Cerebral Circulation.

No. of dogs	Sex	Body wt, kg	Dose of niacinamide, mg/kg i.v.	Cerebral blood flow, ml/100 g/min. (CBF)	Sagittal sinus pressure, mm H_2O	Mean arterial blood pressure, mm Hg (MABP)	Cerebral vascular resistance (CVR)	Cerebral O_2 consumption, ml/100 g/min. ($CMRO_2$)
1	♂	14.0	357	(34.3) 46.8*	(48) 55	(155) 120	(4.54) 2.56	—
2	♂	10.0	350	(48.8) 89.5	(28) 20	(140) 110	(2.86) 1.23	—
3	♀	8.5	375	(53.5) 72.5	(285) 325	(150) 100	(2.76) 1.38	(3.37) 5.80
4	♀	9.0	333	(25.0) 28.0	(400) 200	(125) 55	(5.00) 1.96	(1.95) 2.54
5	♀	9.0	333	(36.2) 51.3	(250) 315	(120) 85	(3.32) 1.66	—
6	♀	10.0	400	(31.2) 39.0	(195) 250	(160) 95	(5.10) 2.43	(2.56) 3.54
7	♀	10.0	300	(46.5) 66.0	(60) 75	(125) 100	(2.68) 1.52	(2.94) 4.85
Mean ± S.E.		10.1 ± 1.6	349.7 ± 55.2	(39.4 ± 7.2) 56.2 ± 12.0	(180.9 ± 61.2) 177.1 ± 55.1	(139.3 ± 22.3) 95.0 ± 16.6	(3.75 ± .71) 1.82 ± .34	(2.71 ± .85) 4.18 ± 1.41
t				3.566	.108	6.646	7.423	3.586
p				<.01	>.9	<.001	<.001	<.05

* The values with and without parentheses were obtained before and 5-15 min. after administration of the drug respectively.

and CVR from 3.75 ± 0.71 to 1.82 ± 0.34 ($P<.001$). However, sinus pressure showed no change.

In contrast with the above mentioned results, the effect of this drug was quite different when small doses (300-500 mg in total) were administered intravenously in 5 dogs. In this series, CBF, sagittal venous pressure, MABP, CVR and $CMRO_2$ showed almost no influence with small doses of this drug.

Discussion. This experiment clearly shows that niacinamide in large doses had distinct effects on CBF, MABP, CVR and MRO_2 of the brain, while no effect could be observed when small doses were administered. Therefore we agree with other investigators(1,2) that this drug in small doses has no effect on cerebral circulation.

Hosoya *et al.*(11) observed that niacinamide in doses of 500 mg orally administered improved dark adaptation (rod adaptation). Although the mechanism of this effect is not clear, it seems not to be due to an increase in cerebral or retinal blood flow, because the dosage of this drug used by these investigators does not increase cerebral blood flow.

Increase in cardiac output does not necessarily cause increase in regional blood flow. This statement can be supported in the case of epinephrine(12); increase in cardiac output by epinephrine results in an increase in liver, skeletal muscle and brain blood flow, but decreases blood flow in the kidney and coronary artery. Niacinamide in large doses, as shown in this experiment, raised cerebral blood flow; therefore, the effect of this drug on cerebral blood flow paralleled its effect on cardiac output. The mechanism of increase in cerebral blood flow caused by large doses of this drug was thought to be by an increase in cardiac output associated with a decrease in cerebral vascular resistance. The low toxicity of this drug should permit its use to increase brain blood flow and metabolism.

Summary. Niacinamide in large doses (3-5 g) administered intravenously increased significantly cerebral blood flow and cerebral oxygen consumption, but decreased mean arterial blood pressure and cerebral vascular resistance in dogs. This drug in small doses had no such effects. The increase in cerebral blood flow was attributed to an increase in cardiac output associated with a decrease in cerebral vascular resistance.

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Adaptation of Coxsackie Virus Strain A-10 to Human Amnion Cells in Tissue Culture.* (26173)

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Following the purification of poliovirus(1) and Coxsackie virus A-10(2), an effort has been made to compare a number of properties of these 2 enteroviruses. Much is known about the biology of the polioviruses, but since Coxsackie virus A-10 could be grown only in suckling mice it was impossible to make a comparative study of the biological behavior of these physically similar viruses. Therefore, attempts were made to adapt Coxsackie virus A-10 to tissue culture. Coxsackie virus A-10 was inoculated onto cultures of cells of human origin (primary amnion(3), HeLa, FL cells(4), and amnion strains A_1-A_7(5)). It was found that the virus multiplied in and caused a cytopathic change in the cells in the primary human amnion cultures but not in the cells of the other cultures tested.

A sample of highly purified, suckling mouse grown Coxsackie virus A-10 was diluted in medium 199 containing 20% lamb serum and sterilized by filtration through a Seitz pad. The sterile filtrate was further diluted to have a titer of about 8.4 x 10^7 suckling mouse LD_{50} per plate of cells, or about 50-100 LD_{50} per cell.

The virus inoculum was placed on 8 petri plates, 4 from each of 2 different preparations of primary human amnion cells (A-847, A-852). Two additional plates from each amnion preparation served as controls and contained only medium 199 with 20% lamb serum.

The control and the inoculated plates were observed daily, and on days 1, 3, 6 and 10, one inoculated plate from each cell preparation was removed from the incubator. The supernatant medium was removed and frozen; the cells on the plates were washed with phosphate buffer, fixed in Bouin's fluid, and stained with haematoxylin. Additional medium (4 ml 199-20% lamb serum) was added to the remaining plates on day 5. Control plates were sampled on days 6 and 10.

On days 1 through 6, no noticeable difference between control and inoculated cells was observed either before fixation or after fixation and staining. On the 8th day, the inoculated plates appeared to differ somewhat from controls, and by the 10th day they were grossly cytopathic. The cells on the control plates appeared to be healthy and well-maintained at 10 days.

The supernatant fluid from each of the 10-day inoculated cultures was diluted 1:5, and each was placed on 2 plates of amnion preparation A-856. The cells of A-856 were grossly cytopathic 3 days after inoculation with the supernatants of both A-847 and A-

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852. An aliquot of the A-847 supernatant was inoculated into suckling mice at log dilutions –2, –3, and –4. None of the mice showed any of the symptoms typical of Coxsackie A-10 infection.

A neutralization test was carried out on the supernatant medium from A-856. Dilutions of 10^{-1} to 10^{-6} were placed on plates of amnion preparation A-857 containing 4 ml 199 with 2% normal monkey serum and 0.1 ml normal rabbit serum, and on plates containing 4 ml 199 with 2% normal monkey serum and 0.1 ml rabbit antiserum to Coxsackie A-10‡. On the third day after inoculation the plates with the Coxsackie A-10 antiserum contained cells of normal, healthy appearance. The plates with no A-10 antiserum were grossly cytopathic at dilutions 10^{-1} through 10^{-4}. The plates with dilutions of 10^{-5} and 10^{-6} became grossly cytopathic after 5 days of incubation. The cytopathic agent was completely neutralized by Coxsackie A-10 antiserum and presumed to be a tissue culture adapted strain of Coxsackie virus A-10 (A-10TC). The virus has been subsequently passaged in many amnion preparations. It shows a cytopathic change within 24 hours and after 11 passages in amnion cells, it was again completely neutralized by Coxsackie A-10 antiserum.

Assays of the tissue culture adapted virus A-10TC, were repeatedly attempted in suckling mice. Titers of <2.5 x 10^2 were occasionally found; however, it appeared that A-10TC had lost most of its mouse virulence.

Samples of A-10TC were inoculated onto a monolayer culture of primary human amnion cells in the routine manner for plaque assays of poliovirus(6,7) and plaques 1-3 mm in diameter developed 3-5 days after inoculation. Titers of 1 x 10^6 to 6 x 10^7 plaque-forming units have been obtained for various stock preparations. Individual preparations of amnion cultures appeared variable in their ability to support plaque formation of A-10TC. Occasionally an amnion yielded no plaques ($<10^{-4}$ titer), whereas simultaneous assays of the same virus sample on other preparations yielded, for example, titers of 1.5 x 10^6, 1.8 x 10^6, and 3.0 x 10^6.

Cytological observations of human amnion cells inoculated with A-10TC showed that the cells undergo the same cytopathic changes observed in human amnion cells after inoculation with poliovirus(8).

This evidence indicates that a strain of Coxsackie virus A-10 has been adapted, or selected, to grow in tissue cultured cells of the human amnion. The virus A-10TC has lost considerable mouse virulence. It is neutralized by Coxsackie A-10 antiserum, and elicits a cytopathic response in the human amnion cells which appears indistinguishable under the light microscope from that caused by infection with poliovirus.

Summary. A tissue culture adapted strain of Coxsackie virus A-10 has been obtained from primary human amnion cells inoculated with virus from a highly purified lot of suckling mouse grown material. The adapted virus (A-10TC) was neutralized by Coxsackie virus A-10 antiserum. It was no longer virulent in mice and caused gross cytopathic changes in primary human amnion cells indistinguishable from those changes caused by the polioviruses. Plaque assays of the virus were obtained on monolayer cultures of primary human amnion cells.

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‡ Coxsackie A-10 rabbit antiserum was kindly supplied by Dr. Natalie Schmidt, California State Public Health Lab., Berkeley.

Inhibitory Effect of Tritiated Thymidine on Regeneration of the Liver in the Young Rat.*‡ (26174)

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Since its introduction, tritiated thymidine (H^3T) has had wide application in the study of proliferating tissue(1). Because it is concentrated in the cell nucleus during synthesis of deoxyribose nucleic acid (DNA) and because most of the radiant energy which it emits is absorbed by the nucleus(2), it is possible that the latter could be adversely affected under some conditions. Cytologic changes attributed to radiation have been noted in HeLa cells grown in fluid containing H^3T(3) and recently in spermatogonia of mice injected with H^3T(4). Evidence is reported here that regeneration of the liver of the young rat is transiently depressed, as indicated by rate of increase of residual liver weight, time sequence of mitotic rate, and presence of abnormal mitoses, by doses of H^3T that are considered to be within the generally accepted tracer range.

Methods. It was determined that after a 12-hour fast the livers of a group of 20 male, Wistar albino rats (60 to 90 g) constituted 4.26 ± 0.10% of their total body weight. Partial hepatectomy(5) was performed on litter mates after a similar fast and the portion of liver removed was blotted on filter paper and weighed. From the ratio of liver weight to body weight previously determined, amount of residual liver was calculated. Times of operation were adjusted so that all animals were killed between 7 and 9 a.m. when mitotic rate is relatively high and constant(6). After operation animals were individually caged and fed Purina chow *ad lib.* H^3T§ (specific activity 360 mc/mM) was given intraperitoneally in doses of 0.25 to 1.0 μc/g body weight (diluted in normal saline solution to a volume of 2.0 ml) at 20 hours following hepatectomy, a time when the liver is maximally synthesizing DNA(7). Control animals were injected with equivalent amounts of nonradioactive thymidine. Groups of animals (6 to 10 per group) were killed at 6, 12, 18, 24, 36, 48 and 72 hours after receiving thymidine. Two additional groups received 1.5 and 2.0 μc H^3T/g body weight at 20 hours after hepatectomy and were killed 24 hours later. A third group was given 1.0 μc H^3T/g body weight every 12 hours, beginning 20 hours after hepatectomy and ending 60 hours later when they were killed. Residual liver was removed, blotted on filter paper, and weighed. Portions of the liver were fixed in 10% neutral buffered formalin for histological preparations. Amount of hepatic regeneration was determined from amount of calculated residual liver at time of operation and amount of liver present at time of death and expressed as per cent increase of residual liver. Corrections were made for changes in body weight. Histological sections were stained by the Feulgen method and with hematoxylin and eosin. Mitotic figures were counted in 6 μ thick, Feulgen-stained sections and expressed as mitoses/100 parenchymal cells. At least 200 mitotic figures or 10,000 nuclei were counted for each animal. Abnormal mitotic figures were defined as bridged anaphase figures(8) and expressed as per cent of total anaphase figures. At least 50 figures were counted for each animal. Autoradiographs were prepared by the dipping technic with melted nuclear track emulsion.‖

Results. Animals receiving nonradioactive thymidine showed an approximately straight-line rate of regeneration for the entire time period studied when corrections were made for changes in body weight (Table I). The mitotic rate reached a peak 26 hours after

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† Life Insurance Medical Research Fund Fellow.

‡ Portions of this work were read before the Am. Assoc. for Study of Liver Disease, Chicago, Ill., Nov. 5, 1958.

§ Schwarz BioResearch, Mt. Vernon, N. Y.

‖ Eastman Kodak Co., Rochester, N. Y. Nuclear Track Emulsion, NTB-3.

TABLE I. Per Cent Increase in Residual Liver as a Function of Amount of Tritiated Thymidine Given.

Time (hr) after hepatectomy	μc H^3T/g body wt 0	.25	.5	1.0	1.5	2.0	5.0*
6†	5.9 ± 1.0‡	—	—	—	—	—	—
12	10.1 ± 1.3	—	—	—	—	—	—
20	18.4 ± 1.6	—	—	—	—	—	—
26	22.0 ± 1.4	23.2 ± 1.6	20.2 ± 1.5	17.4 ± 1.5	—	—	—
32	26.0 ± 1.2	24.6 ± 1.7	22.4 ± 1.8	18.0 ± 1.6§	—	—	—
38	30.4 ± 1.3	31.5 ± 1.7	27.6 ± 1.9	22.2 ± 1.8§	—	—	—
44	39.0 ± 1.5	42.3 ± 1.8	35.4 ± 1.9	26.2 ± 2.0§	22.8 ± 2.2§	21.5 ± 2.5§	—
56	43.0 ± 2.0	45.1 ± 2.2	39.5 ± 2.0	34.2 ± 2.4	—	—	—
68	52.1 ± 2.6	53.0 ± 3.1	48.7 ± 2.7	43.5 ± 2.5	—	—	—
80	59.6 ± 2.5	62.2 ± 3.2	54.6 ± 3.4	52.1 ± 2.8	—	—	40.8 ± 3.4§
92	69.7 ± 3.0	71.6 ± 3.4	64.2 ± 3.2	65.6 ± 2.4	—	—	—

* Given in 5 divided doses, 1 μc/g body wt every 12 hr beginning at 20 hr after hepatectomy.
† Each group contains 6-10 animals.
‡ Mean ± stand. error of mean.
§ Indicates P equal to or greater than .01 when compared to control value.

hepatectomy, then declined quickly (Table II). In control animals at 26 hours after hepatectomy, 4.7 ± 1.0% of anaphase mitotic figures were abnormal (Table III).

In animals receiving 1.0 μc H^3T/g body weight, replacement of liver mass at 12, 18 and 24 hours after injection was significantly depressed. The inhibition was transitory and by 36 hours after injection the weights of residual liver were not significantly different from the controls (Table I). In animals receiving 1.5 and 2.0 μc H^3T/g body weight, replacement of liver mass was also depressed 24 hours after injection, the only time interval measured. Six hours after injection of 1.0 μc H^3T/g body weight the mitotic rate was no greater in hepatectomized animals than in nonhepatectomized controls. A rebound peak was reached 24 hours after injection and the number of mitoses continued to be significantly elevated above control values for the entire period under study (Table II). At 44 hours after hepatectomy, 28.2 ± 4.8% to 33.4 ± 7.9% of the anaphase mitotic figures were abnormal in animals receiving 1.0 to 2.0 μc H^3T/g body weight (Table III). Autoradiography showed 70 to 90% of the abnormal mitotic figures to be radioactive.

In animals receiving 1.0 μc H^3T/g body weight every 12 hours between 20 and 80 hours after hepatectomy, the residual liver was significantly smaller than in the controls,

TABLE II. Mitotic Rate in Residual Liver as a Function of Amount of Tritiated Thymidine Given.

Time (hr) after hepatectomy	μc H^3T/g body wt 0	.25	.5	1.0	1.5	2.0	5.0*
0†	.15 ± .10‡	—	—	—	—	—	—
6	.20 ± .07	—	—	—	—	—	—
12	.40 ± .15	—	—	—	—	—	—
20	.90 ± .20	—	—	—	—	—	—
26	3.31 ± .41	3.10 ± .34	2.44 ± .32	.80 ± .20§	—	—	—
32	2.40 ± .30	2.56 ± .40	2.85 ± .44	.90 ± .23§	—	—	—
38	1.52 ± .31	1.82 ± .31	2.20 ± .40	1.92 ± .32	—	—	—
44	1.10 ± .15	1.43 ± .22	1.60 ± .34	3.79 ± .45§	3.20 ± .55§	3.00 ± .60§	—
56	.80 ± .13	1.02 ± .20	1.20 ± .25	2.60 ± .32§	—	—	—
68	.70 ± .15	.85 ± .16	.90 ± .20	2.23 ± .25§	—	—	—
80	.60 ± .12	.70 ± .10	.75 ± .15	2.02 ± .22§	—	—	2.40 ± .42§
92	.50 ± .15	.55 ± .12	.70 ± .13	1.65 ± .20§	—	—	—

* Given in 5 divided doses, 1 μc/g body wt every 12 hr beginning at 20 hr after hepatectomy.
† Each group contains 6-10 animals.
‡ Mean ± stand. error of mean.
§ Indicates P equal to or greater than .01 when compared to control value.

TABLE III. Per Cent Abnormal Anaphase Figures as a Function of Amount of Tritiated Thymidine Given.

Time (hr) after hepatectomy when anaphase figures counted	μc H^3T/g body wt						
	0	.25	.5	1.0	1.5	2.0	5.0*
26†	4.7 ± 1.0‡	5.6 ± 1.4	9.6 ± 3.7	—	—	—	—
44	—	—	—	28.2 ± 4.8§	30.8 ± 5.2§	33.4 ± 7.9§	—
80	—	—	—	—	—	—	43.5 ± 7.8§

* Given in 5 divided doses, 1 μc/g body wt beginning at 20 hr after hepatectomy.
† Each group contains 6-10 animals.
‡ Mean ± stand. error of mean.
§ Indicates P equal to or greater than .01 when compared to control value.

the mitotic rate was much higher, and 43.5 ± 7.8% of the anaphase figures were abnormal.

Discussion. The regenerating liver of the rat subjected to partial hepatectomy is a mitotically active tissue, surpassing at certain stages in its growth any other normal adult tissue, embryonic tissue, and many neoplasms (9). Cater, *et al.*(10) have shown the regenerating liver to be very sensitive to radiations. They detected mitotic abnormalities with the smallest dose of x-rays used in their study (150 r).

Irradiation of the cell during certain stages of the mitotic cycle dissociates DNA synthesis from mitosis by blocking the latter(11). In our animals, the radioactive material was incorporated during synthesis and labeled cells were apparently prevented from entering mitosis for a variable period of time. The depression of cell division was also associated with some slowing of regeneration of liver mass as compared to controls. About 12 to 24 hours after the rats received the injection of H^3T, the cells began to recover their mitotic ability but did not reach a peak near that of the controls until some 12 hours later. The mitotic rate remained elevated at a time when that in control animals had already returned to normal. This rebound phenomenon is typical of mitotically active tissue in response to sublethal doses of radiation(8). Associated with the reappearance of mitoses were an increased number of mitotic abnormalities as indicated by anaphase bridges. Brues(8) has discussed the difficulty of using bridged figures as an indication of radiation damage in liver since normally there is an appreciable incidence of mitotic aberrations associated with regeneration. This normal incidence we found in the controls, but the level in the treated animals was clearly higher and significantly elevated, as has been found by Albert(12) in radiation depression of hepatic regeneration in mice and by Cater, *et al.* (10) in rats. Between 70 and 90% of the abnormal mitoses were radioactive, therefore the nonradioactive abnormal mitoses were similar in number in both groups.

After reestablishment of mitoses in the treated group, liver mass increased until 72 hours after injection when it reached control levels. Animals receiving 1.0 μc H^3T/g body weight have been followed for long intervals and no further abnormality has been noted. Apparently the damage is transitory and the irradiated hepatic cells either recover completely or die and are replaced by normal cells.

Such changes are extremely important in attempting to study temporal relationships of DNA synthesis and mitosis. For instance, we have observed then when 0.2 to 0.3 μc H^3T/g body weight is given to rats at intervals after hepatectomy and shortly before they are killed, rate of appearance of labeled cells and mitotic rate parallel each other with the former preceding the latter by about 6 to 8 hours. However, when 1 μc H^3T/g body weight or more is given the rate of appearance of mitoses is delayed for 6 to 12 hours and the curve is skewed to the right. Obviously, it is necessary to use a dose of H^3T that does not cause mitotic inhibition for a study of this type to have validity. Doses of 0.3 μc H^3T/g body weight in rats or mice give adequate labeling for autoradiography, and to prevent the complication of possible radiation damage, larger amounts should not be used. This is especially important in studies in which subsequent development or movement of the labeled cells is to be followed.

Summary. Tritiated thymidine, when given to young male rats 20 hours following partial hepatectomy in doses of 1.0 μc/g body weight or greater, causes a transient inhibition of regeneration as mirrored in rate of increase of residual liver mass, mitotic rate, and incidence of abnormal mitotic figures. The importance of this effect in tracer studies is obvious.

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Action of Angiotensin on Isolated Guinea Pig Ileum. (26175)

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Earlier studies(1,2) with angiotensin (hypertensin, angiotonin) indicated a direct spasmogenic action on smooth muscle. A recent report(3) suggests that on rabbit and guinea pig ileal strips, angiotensin may have an indirect action in producing contraction. The present study attempts to delineate the site of action of angiotensin in guinea pig ileum by use of pharmacologic blocking agents. The results suggest that angiotensin produces contraction by acting on the post-ganglionic cholinergic mechanism. They also indicate that care should be taken in interpreting such results and that a number of blocking agents of each class should be examined over a wide range of concentrations before conclusions are reached.

Materials and methods. Freshly prepared strips of guinea pig ileum were suspended in duplicate 10 ml baths of aerated Tyrode's solution maintained at 37°C. The responses were recorded on soot-paper by frontal writing levers. Maximal contractions were induced at 4-minute intervals by exposing the tissues to 0.05 γ/ml of angiotensin* for 60 seconds and to 1 γ/ml of nicotine for 30 seconds. The antagonists were added midway between contractions allowing 2-minute exposure of the tissue. Three or more concentrations of each antagonist were studied to obtain dose-response curves. The EC_{50}'s, concentration effecting a 50% inhibition of the contractions, were obtained from graphic plots. Each of the antagonists was studied on 6 ileal strips. The results are presented in Table I.

Results and discussion. The data establish that the spasmogenic actions of angiotensin and nicotine on the ileum are effectively blocked by low concentrations of atropine and morphine. Emmelin and Feldberg (4) have shown that atropine effectively blocked the action of nicotine on the gut while Schaumann(5) and Paton(6) have

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TABLE I. Effect of Autonomic Blocking Agents on Angiotensin and Nicotine-Induced Contractions of Guinea Pig Ileum.

	Spasmogen			
	Angiotensin		Nicotine	
Antagonist*	Avg EC_{50}	γ/ml range	Avg EC_{50}	γ/ml range
Atropine	.02	(.01 – .04)	.01	(.003– .03)
Morphine	.06	(.002– .16)	.19	(.004– .96)
Mecamylamine	28	(16 – 41)	.11	(.05 – .19)
Pempidine	306	(115 –320)	.10	(.06 – .14)
Pentolinium	>500		.33	(.11 – .68)
Hexamethonium	>500		.82	(.47 –1.3)

* As base or ion.

demonstrated that morphine blocks the effects of cholinergic stimulation by preventing release of acetylcholine. With only this information, one would be tempted to classify angiotensin with nicotine as having ganglion-stimulating properties. These results with atropine do not confirm those of Collins(7); however, his use of crude angiotensin may have resulted in spasmogenic impurities not affected by atropinization. When 4 ganglionic blocking agents were examined, it was shown that all blocked nicotine at low concentrations; however, only mecamylamine (3-methylaminoisocamphane) and pempidine (1:2:2:6:6-pentamethylpiperidine) exhibited blocking activity against angiotensin and then only at concentrations several hundred-fold higher than their nicotine EC_{50}'s. This indicates that the inhibition observed was probably not due to ganglionic blockade. The results obtained, however, may be related to a reported local anesthetic effect produced by mecamylamine on the isolated rabbit ileum (8).

The lack of blocking effect observed with high concentrations of hexamethonium (1,6-bis-[trimethylammonium]-hexane) and pentolinium (pentamethylene-1:5-[bis-1-methylpyrrolidinium]) causes angiotensin to resemble more closely the action of serotonin on the guinea pig ileum as reported by Rocha e Silva *et al.*(9). The inability of the latter 2 ganglionic blocking agents to penetrate intracellularly may be related to their inactivity. If this latter possibility is fact, it implies that angiotensin is acting at an intracellular site.

These results indicate that angiotensin is acting at some point on the post-ganglionic cholinergic mechanism to produce contraction of guinea pig ileal smooth muscle and confirm the earlier report(3) in which botulinium toxin was used to show an indirect effect of angiotensin. This proposed site of action is unique only for the guinea pig ileum since it is well established that angiotensin acts directly on vascular smooth muscle(1).

Conclusions. 1. Atropine and morphine effectively block angiotensin and nicotine-induced spasm on the isolated guinea pig ileum. 2. Mecamylamine, pempidine, pentolinium and hexamethonium blocked nicotine-induced spasms; however, only mecamylamine and pempidine blocked angiotensin spasms, but very high concentrations were required. 3. It is proposed that angiotensin acts on the post-ganglionic cholinergic mechanism of the ileum and that its site of action is probably peripheral to the ganglion.

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Effect of Certain Compounds on *in vitro* Degradation of Serotonin by Ceruloplasmin.* (26176)

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It was recently demonstrated in this laboratory that *in vitro* degradation of serotonin by ceruloplasmin is strongly inhibited by both isoniazid and iproniazid(1). This finding may be of importance in connection with the psychopharmacologic and other clinical effects of these compounds. Moreover, it may have significance with regard to a concept which postulates that fibrosis may result from an imbalance between serotonin and serotonin degradation mechanisms at the tissue level(1,2,3). In view of these and other considerations, it became of interest to determine the *in vitro* effect of a wide range of other compounds on this pathway of serotonin oxidation.

Accordingly, tests were performed with selected reducing, sulfhydryl containing, and metal binding compounds; with hydrazine derivatives and amine oxidase inhibitors; and with a number of antihypertension and psychopharmacologic agents. The findings form the basis of this report.

Materials and methods. Initial titration tests with purified preparations of ceruloplasmin confirmed the activity of this enzyme over a wide pH range. Although optimal activity was at pH 6.0, it was elected to perform these *in vitro* tests at a more closely physiological pH, that is, 7.4. Accordingly, serial 2-fold dilutions of ceruloplasmin were made in physiological saline solution. The enzyme concentration which was found to metabolize between 65% and 80% of the substrate (300 μg serotonin-creatinine sulfate) was used in the final test system. This degree of enzyme activity was found to be present in a 1:8 dilution of the human ceruloplasmin employed. Dilutions of 3 different preparations of ceruloplasmin were used. These were designated DWZ 37, DWZ 42, and DWZ 44, and had been prepared by the Kabi Co., Stockholm, Sweden[†]

Procedure. To a 20 ml beaker were added 1.0 ml of ceruloplasmin solution; 1.0 ml of 0.5 M phosphate buffer, pH 7.4; and 0.5 ml of the solution containing the substance to be tested for inhibition of the reaction. The beakers were pre-incubated for 15 minutes on a Dubnoff metabolic shaker. Solutions of the test substances were freshly prepared and placed in the test system within 10 minutes of preparation. Final concentration in the incubation mixture was 4.9 x 10^{-4} M for serotonin. Incubation was continued for 1 hour, and determination for residual serotonin was made by the colorimetric method of Udenfriend, *et al.*(4). Controls included tests with boiled ceruloplasmin, and, for color interference, recovery of serotonin after incubation with the respective test substance in absence of enzyme.

The following substances were tested for reaction inhibition at a final concentration of 1 x 10^{-3} M: sodium metabisulfite, ascorbic acid, glutathione, British anti-lewisite (BAL), cysteine, methionine, D, L penicillamine, sodium cyanide, and ethylene diamine tetracetic acid (EDTA). Among the hydrazine derivatives and among oxidase inhibitors tested at the same concentration were: amphetamine, procaine amide, iproniazid,[‡] isoniazid,[‡] isocarboxazid[‡] (Marplan),® hydralazine[§] (Apresoline),® Compound Ba-20523[§] (an analog of hydralazine), thiocarbohydrazide, semicarbazine, and aminoguanidine. Hydrazine hydrate, phenylhydrazine, 2-4 dinitrophenylhydrazine, 1-acetyl-2-phenylhydrazine, and diphenylthiocarbazone were employed in a final dilution of 1 x 10^{-5} M be-

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[†] The ceruloplasmin used in this study was generously made available by the Wyeth Laboratories, Radnor, Pa.

[‡] Kindly provided by Hoffmann-LaRoche, Nutley, N. J.

[§] Contributed by Ciba Pharmaceutical Products, Summit, N. J.

cause of color interference at greater concentrations.

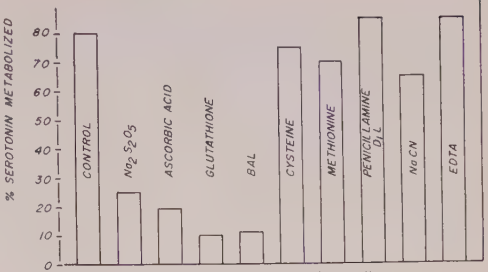
The antihypertensive and psychopharmacologic substances tested at a final concentration of 1 x 10^{-4} were Su-7057§ (a thiazide diuretic), Su-5879§ (Esidrix,® hydrochlorothiazide), Su-6835§ (an analog of hydrochlorothiazide), reserpine, Su-3118§ (syrosingopine, Singoserp,® a synthetic analog of natural Rauwolfia alkaloids), Su-5171§ (an isomer of syrosingopine), chlorisodamine chloride§ (Esidrix),® hexamethonium bromide, tetraethylammonium chloride, methylphenidate hydrochloride§ (Ritalin),® and Ro-50690‡ (Librium).® Su-5864§ (guanethidine, an antihypertensive agent), and Su-6187§ (a thiazide diuretic) were employed in a final concentration of 0.0285 mg/ml.

All of the reagents were chemically pure substances except for BAL which was prepared from a solution containing peanut oil and benzyl benzoate.

Results. The majority of test findings are presented in Fig. 1-3. At pH 7.4, an inhibitory effect on *in vitro* degradation of serotonin was exhibited by sodium metabisulfite, ascorbic acid, glutathione, BAL, isoniazid, iproniazid, isocarboxazid, hydralazine, Ba-20523, thiocarbohydrazide, and hydrazine hydrate. An effect about equal to the latter was also noted with compounds Su-3118 (Singoserp) and its isomer, Su-5171. Lesser changes in activity were found in presence of reserpine, tetraethyl ammonium chloride, and Su-7057, as well as with phenylhydrazine, 2-4-dinitrophenylhydrazine, and 1-acetyl-2-phenylhydrazine.

None of the other substances appeared to alter the reaction rate under the conditions of testing.

Discussion. It is evident from the foregoing results that enzymic degradation of serotonin by ceruloplasmin is markedly inhibited at physiologic pH by a number of widely differing compounds. Reducing agents such as sodium metabisulfite, ascorbic acid, glutathione, and BAL are particularly active. In this connection, it may be noted that Holmberg and Laurell demonstrated reduction of ceruloplasmin to a colorless compound by sodium hydrosulfite, ascorbic acid, hydroxylamine, and thioglycolic acid(5), while ascorbic acid was further shown to diminish enzyme activity(6). This property of ascorbic acid has been utilized by R. B. Davis to block the destruction of serotonin present in specimens of clotted blood(7). Holmberg and Laurell also found that potassium cyanide inhibited oxidation of paraphenylene diamine, especially on the acid side of pH 7(6). This is in contrast to the minimal effect of



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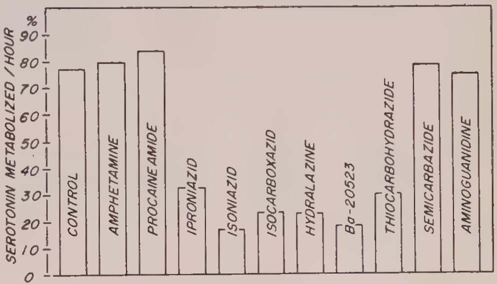

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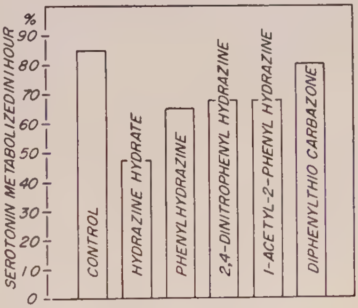

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FIG. 1. *In vitro* effect of certain reducing, sulfhydryl containing, and metal binding agents on serotonin degradation by ceruloplasmin.

FIG. 2. Effect of indicated compounds on *in vitro* degradation of serotonin by ceruloplasmin preparation DWZ 37.

FIG. 3. Effect of certain hydrazine derivatives on *in vitro* degradation of serotonin by ceruloplasmin preparation DWZ 42.

NaCN as observed in this study at pH 7.4.

It is of interest that EDTA and penicillamine did not inhibit the reaction. It appears, therefore, that the copper of ceruloplasmin was not chelated. R. P. Davis observed a similar failure to chelate enzyme metal in studies with carbonic anhydrase(8). He suggested that the lack of chelation of carbonic anhydrase zinc might reflect a more internal location of this metal in the carbonic anhydrase molecule than is the case for other zinc enzymes where chelation results in competitive inhibition and where a superficial location is necessary in the substrate binding function of those enzymes. It seems reasonable to consider that a similar relationship probably exists with regard to the copper of ceruloplasmin.

With regard to the hydrazine derivatives, Martin, *et al.*(9) demonstrated that oxidation of 5-hydroxytryptamine by serum from normal and pregnant humans was inhibited by .003 M iproniazid. These workers suggested that the enzyme involved was ceruloplasmin and further noted that this offered a potential degradation pathway for serotonin transformation other than the monoamine oxidase system. The present studies with purified ceruloplasmin revealed not only iproniazid, but also isoniazid, hydralazine, an analog of hydralazine (Ba-20523), isocarboxazid, and thiocarbohydrazide are inhibitory, as is hydrazine hydrate to a lesser extent. On the other hand, semicarbazide, aminoguanidine, and diphenylthiocarbazone were without effect. Thus, the presence of the basic hydrazine nucleus in a compound is not sufficient in itself to produce inhibition of this reaction.

Several of the test substances are classified by some as "monoamine oxidase inhibitors," while others are termed "diamine oxidase inhibitors." Among the former are amphetamine, procaine amide, iproniazid, and isocarboxazid. It can be seen from the results that some inhibited ceruloplasmin activity whereas others did not. Similarly, thiocarbohydrazide, a "diamine oxidase inhibitor," markedly depressed ceruloplasmin oxidation, while semicarbazide and aminoguanidine were ineffective.

Hydralazine, and its analog, Ba-20523, are both potent diamine oxidase inhibitors. Hydralazine also strongly inhibits monoamine oxidase, but its analog is about 30 times less active in this regard(10). Both substances were markedly inhibitory to ceruloplasmin activity. The findings, therefore, fail to show correlation with either monoamine or diamine oxidase inhibition.

Numerous reports have dealt with the relationship of serotonin and other amines, such as norepinephrine, to central nervous system activity. The psychopharmacologic effects of monoamine oxidase inhibitors, for example, are believed to be mediated through inhibition of this pathway of destruction of brain serotonin and/or norepinephrine(11). Recent observations by Sulser and Brodie(12) underscore the complexity of interaction of these mechanisms at the brain tissue level. Their findings suggest that the tranquilizing action of reserpine is not related to brain norepinephrine loss but rather to a change in level of brain serotonin.

Our findings indicate an even more complex relationship is probably involved. Thus, reserpine and 2 of its analogues (syrosingopine and its isomer, Su-5171) were found to have some inhibitory effect on degradation of serotonin by ceruloplasmin. Degree of inhibition was not as striking as that obtained with hydralazine, its analog (Ba-20523), isoniazid, and isocarboxazid. Nevertheless, it should be apparent that modifying effects on pathways of serotonin degradation other than monoamine oxidase must also be taken into account when interpreting the clinical actions of various compounds.

Of the remaining substances tested in this study, only TEAC and Su-7057 exhibited some inhibitory effect on the reaction test-system. That TEAC produced inhibition despite the presence of the chloride ion is of interest since Holmberg and Laurell(13) showed that certain monovalent anions, including Cl^- and Br^-, had an accelerating effect on this enzymic process. Hexamethonium bromide, however, was without evident effect. Su-7057, which showed slight inhibition, was the only thiazide derivative tested to do so. The reason for this finding is not known.

Summary. In an *in vitro* test system at pH 7.4 it was found that degradation of serotonin by ceruloplasmin was markedly inhibited by sodium metabisulfite, ascorbic acid, glutathione, BAL, isoniazid, iproniazid, hydralazine, an analog of hydralazine (Ba-20523), isocarboxazid, thiocarbohydrazide, hydrazine hydrate, syrosingopine (Su-3118) and its isomer, Su-5171. Lesser effects were produced by reserpine, tetraethylammonium chloride, and a thiazide diuretic (Su-7075). A number of other sulfhydryl containing, chelating, anti-hypertension, and psychopharmacologic agents were without effect in the test system employed. It was not possible to correlate reaction inhibition with simple presence of sulfhydryl groups, the hydrazine nucleus, or with monoamine oxidase and diamine oxidase inhibition.

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Pyridoxal Phosphate in Plasma and Leukocytes in Patients with Leukemia and Other Diseases.* (26177)

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The concentration of pyridoxal phosphate, the coenzymatically active form of Vit. B_6, can be estimated in leukocytes and plasma using a modification of the manometric method of Umbreit *et al.*(1). Previously, this has been done in the blood of normal (2,3,4) and pregnant subjects(3,4) and in the cord blood of new born babies(3,4). In addition, load tests were performed in normal controls and in pregnant women with measurement of the B_6 content of plasma and leukocytes following oral administration of pyridoxine hydrochloride(4). The present communication deals with estimation of pyridoxal phosphate in the blood and plasma of patients with chronic and acute leukemia and various other diseases.

Method. For simultaneous estimation of pyridoxal phosphate in leukocytes and plasma, 5 ml of venous blood were collected in a tube containing about 0.002 g of sodium versenate. The assay method is based on coenzyme function of pyridoxal phosphate in a tyrosine decarboxylase system according to Boxer *et al.*(2) with minor changes as previously described(4). Streptococcus fecalis was obtained from the Dept. of Bacteriology, Rutgers Univ., New Brunswick, N. J. Leukocytes were isolated using freeze-dried phytohemagglutinin prepared from navy beans. The phytohemagglutinin does not contain any measurable B_6. The samples were usually

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* Supported by Vitamin Fn.

TABLE I. Pyridoxal Phosphate Values in Plasma and Leukocytes in Normal Controls and Various Disease Groups.

Group	No. of cases	Plasma, mγ/ml		Leukocytes, mγ/million cells		% of cases below		P	
		Avg	Range	Avg	Range	4 mγ/ml plasma	0.13 mγ/mil leuko.	Plasma	Leukocytes
Normal	40	9.6 ± 2.8	5.2-33	.22 ± .05	.14-.36	0	0		
Fibroid uterus	12	6.0 ± 5.2	1.1-19.4	.22 ± .10	.05-.44	42	8	0	0
Cancer	23	4.3 ± 4.1	.0-16.2	.21 ± .02	.07-.68	50	26	0	0
Cardiac	26	7.1 ± 3.8	2.0-16.5	.25 ± .08	.05-.38	19	8	0	0
G.I. pathology	25	6.2 ± 6.2	.7-23.1	.26 ± .11	.08-.62	44	12	0	0
Respiratory diseases	20	7.5 ± 7.6	.7-30.2	.24 ± .10	.14-.54	35	0	0	0
Diabetes mellitus	21	6.3 ± 3.8	1.1-30.2	.25 ± .11	.10-.54	33	9	0	0
Liver cirrhosis	11	4.6 ± 3.9	.7-12.5	.22 ± .13	.08-.55	50	18	0	0
Obstructive jaundice	22	5.0 ± 4.8	.0-16.2	.18 ± .14	.02-.68	60	41	0	0
Infectious hepatitis	9	6.6 ± 5.4	1.6-19.7	.24 ± .22	.00-.59	33	44	0	0
Nutritional anemia	4	2.4 ± 3.0	1.1- 3.8	.21 ± .20	.07-.52	100	50	0.10	0
Pernicious "	5	3.7 ± 3.2	.0- 7.2	.22 ± .11	.04-.31	50	20	0	0

obtained in the morning. The subjects were either fasting or had received a light breakfast. It was ascertained in control experiments that breakfast had no influence on Vit. B_6 level of plasma or leukocytes. No vitamin preparation had been taken by any of the individuals studied for at least 2 months. Determinations were done in duplicate within 12 hr after samples had been obtained. Even at 4°C some loss of Vit. B_6 occurred after 24 hr in plasma and after 48 hr in leukocytes. Duplicate determinations for plasma showed an error of ± 0.5 mγ/ml and for leukocytes of ± 0.03 mγ/million cells.

Results. Table I shows the results obtained in normal controls and in hospital patients excluding those suffering with leukemia. In normal controls, pyridoxal phosphate concentration ranged between 5.2 and 33 mγ/ml with an average of 9.6 mγ in plasma, and between .14 and .36 mγ/million cells with an average of .22 mγ in the leukocytes. Patients were divided, for convenience, into various groups as listed in the Table. There were wide variations of B_6 content of plasma and leukocytes in all groups and, therefore, standard deviations were great. Average values for leukocytic pyridoxal phosphate were close to those of the normal group. On the other hand, average plasma values were considerably lower in several of the patient groups, particularly those with nutritional anemia, pernicious anemia, cancer of various sites and liver cirrhosis. However, a statistically significant difference was found only in patients with nutritional anemia.

The Table also lists the percentage of cases in each group in which plasma contained less than 4 mγ/ml and leukocytes less than .13 mγ/million cells. These amounts were lower than the lowest found in the normal group. In patients with obstructive jaundice, infectious hepatitis and nutritional anemia, between 40 and 50% of the patients had such low leukocyte values. Plasma estimations revealed values of less than 4 mγ/ml in many of the patients regardless of the type of disease for which they had been admitted to the hospital.

Table II lists findings in cases of leukemia as well as those of normal bone marrow and lymphocyte suspensions from surgically removed tonsils. Leukemias were grouped into chronic myeloid, chronic lymphatic and acute. Total white counts in the patients with chronic myeloid leukemia varied between 28,000 and 500,000, with chronic lymphatic, between 5,200 and 900,000, and with acute leukemia, 3,200 and 125,000 cells per cc. Average leukocyte values were between .06

TABLE II. Pyridoxal Phosphate Values in Plasma and Leukocytes in Leukemias and in Normal Bone Marrow and Tonsils.

Group	No. of cases	Plasma, mγ/ml Avg	Plasma, mγ/ml Range	Leukocytes, mγ/million cells Avg	Leukocytes, mγ/million cells Range	% of cases below 4 mγ/ml plasma	% of cases below 0.13 mγ/mil leuko.	P Plasma	P Leukocytes
Leukemia									
Chronic myeloid	7	3.1 ± 2.2	1.5-6.3	.07 ± .04	.03-.16	60	87	.05	.01
" lymphatic	11	4.9 ± 2.6	1.9-8.8	.06 ± .04	.00-.14	66	91	.10	.01
Acute	15	3.5 ± 2.7	.0-7.2	.09 ± .04	.03-.16	60	80	.05	.01
Normal bone marrow	5			.20 ± .11	.09-.38				
Tonsils	10			.19 ± .07	.09-.31				

and .09 mγ/million cells and for plasma, between 3.1 and 4.9 mγ/ml. The difference between the patients suffering from leukemia and the normal control group is statistically significant. Correspondingly between 80-91% of all cases showed leukocytic values lower than .13 mγ/million cells and two-thirds showed plasma values lower than 4 mγ/ml. In 5 bone marrows of subjects either healthy or with minor illnesses, the white cells contained between 0.09 and 0.38, average .20 mγ/million cells of pyridoxal phosphate. Lymphocytes obtained from homogenates from tonsils from 10 otherwise healthy children contained between .09 and .31, average .19 mγ/million cells of pyridoxal phosphate. Thus, in both instances, considerably greater amounts of B_6 were found in normal bone marrow cells and tonsillary lymphocytes.

Comment. These findings show that the average amount of B_6 in leukocytes and plasma is not statistically different in patients with various diseases as compared to those of normal controls with the exception of patients with leukemia and in the case of the plasma, those with nutritional anemia. A considerable number of nonleukemic patients, however, had lower amounts of B_6 than the lowest found among controls. This may be due in individual cases either to a decreased intake or an increased need for this essential vitamin. Similarly, Boxer *et al.*(2) found in 7 out of 36 patients with manifest arteriosclerotic disease very low amounts of leukocytic B_6.

The consistent depression of leukocytic B_6 of leukemic patients cannot be due to the relative immaturity of the leukemic cells since many of the leukocytes in the blood of patients with chronic myeloid and most with lymphatic leukemia were mature. Furthermore, cells from normal bone marrow contain a significantly higher amount of B_6 than those from patients with chronic myeloid leukemia. Likewise, lymphocytes obtained from tonsils contain more pyridoxal phosphate than do leukemic lymphocytes. It is interesting to note that so far only leukocytes in pregnant women at term revealed a comparable depression of leukocytic B_6 attributable to depletion of the maternal tissues by the growing fetus(3,4). A marked abnormality in B_6 metabolism of a different, as yet unknown, etiology has been recently described in several neoplastic processes. Thus total Vit. B_6 content of hepatomas induced by butter yellow in rats is considerably lower in the tumor as compared to the normal liver (5). Haggerty and Sullivan(6) found not only relatively small values of B_6 in a transplanted sarcoma in CAF_1 mice, but in addition, a progressive decrease in Vit. B_6 content of the tumor during its growth with a simultaneous accumulation of kynurenin. In human subjects, Musajo *et al.*(7) isolated kynurenin and 3-hydroxykynurenin from the urine of patients with myeloid and lymphatic leukemia and Hodgkin's disease.

Summary. The amount of coenzymatically active Vit. B_6 was determined in plasma and leukocytes in patients with various diseases. Values for plasma and leukocytes varied widely in all groups examined. A statistically significant decrease of plasma B_6 was found only in patients with nutritional anemia and various forms of leukemia. However, the amount of pyridoxal phosphate was very low in individual patients with various other diseases. This may indicate a decreased intake of vitamin in some hospitalized patients or an increased need for it. B_6 values in leukocytes were consistently decreased in patients with various forms of leukemia. In contrast, cells from normal bone marrow and from tonsils showed significantly higher amounts of pyridoxal phosphate. The findings are discussed with reference to a specific disturbance in B_6 metabolism in some neoplastic conditions.

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Liver Ketogenesis (3). High Fat Feeding on Acetoacetate Production in Liver Slices, Utilization by Kidney Slices. (26178)

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Nath *et al.*(1-3) have reported that continued injection of acetoacetate to normal animals causes disturbances in carbohydrate metabolism and it has been postulated that gradual accumulation of acetoacetate in blood and tissues is one of the factors responsible for development of diabetes. Such accumulation may result either from overproduction of acetoacetate in the liver or its decreased utilization by the extra-hepatic tissues.

The authors(4) have reported that the hydrolyzed product of glucose cycloacetoacetate (GCA), a crystalline substance obtained by condensation of glucose with acetoacetate, prevents overproduction of acetoacetate by liver slices and increases its utilization by kidney slices when fed to rats along with a high carbohydrate diet. High fat diet has also been known to cause metabolic changes in rats, including diminished rate of lipogenesis (5), glycogenesis(6) and glucose utilization (7). Therefore, the effect of high fat feeding on liver ketogenesis and the influence of hydrolyzed GCA feeding with high fat diets is reported here. Desiccated thyroid and thiouracil were also mixed in the diet of 2 groups of animals to study their additional influence on liver ketogenesis and acetoacetate utilization by kidney slices.

Methods. In view of the relatively rapid response of young rats to thyroid and thiouracil feeding(8), male albino rats weighing 75-100 g and growing steadily on a standard diet were used. These rats were divided into 8

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groups of 6 rats each. Rats in Group I were maintained on high carbohydrate diet (Normal). All animals in other groups were fed high fat diet.

COMPOSITION OF DIETS

Constituents	Normal (%)	High fat (%)
Wheat flour	10	13
Sucrose	50	15
Casein	20	25
Ground-nut oil	9	34
Cod liver oil	1	1
Salt mixture	4	4
Yeast powder	4	6
Marmite	2	2

Rats in Group IIa were fed high fat diet for 19 weeks. Desiccated thyroid and thiouracil (.3% of diet by wt.) was mixed in the diet fed to rats in Groups III, VI and IV, VII respectively. Freshly hydrolyzed GCA (.8%) was fed along with diet to Groups V, VI and VII.

Tissue preparation and analytical methods. At the end of feeding period of 7 weeks (19 weeks in case of Group IIa), rats were sacrificed by stunning and decapitation. Liver and kidneys were removed immediately and placed in ice-cold saline. Tissue preparations were done as described by Umbreit *et al.*(9). Liver and kidney slices were then cut with razor blade, weighed in microbalance and suspended in conventional Warburg vessels containing 3 ml of Krebs' Ringer Phosphate buffer, pH 7.2. Vessels were gassed for one minute with oxygen, then incubated for 2 hr with constant shaking. At the end of incubation period, acetoacetate was estimated from the media according to the manometric method of Edson(10). Liver glycogen was estimated at the beginning, by the method of Good *et al.*(11), and blood sugar by that of Hagedorn and Jensen(12). GCA was prepared according to the method of West(13) as modified by Nath *et al.*(14). GCA was hydrolyzed with 2N HCl in boiling water bath for 12 min. and extracted with ether to remove resinous materials formed. Aqueous solution was adjusted to pH 7.2 by adding the requisite amount of 2N NaOH. Total fat content of liver was extracted thrice with hot alcohol-ether mixture (1:1). This extract was evaporated to dryness and remaining solids were reextracted with petroleum ether and evaporated in an air oven to dryness till constant weight is reached. From this the amount of fat was calculated per g of wet liver.

TABLE I. Oxygen Uptake and Acetoacetate Produced by Liver and Kidney Slices. 6 rats in each group except Group IIa with 5 rats. Krebs' Ringer Phosphate Buffer pH 7.2, incubation temperature 37.5°C; oxygen as gas phase. (Avg values with stand. dev.)

	Oxygen uptake in μl/100 mg wet wt of tissue/2 hr					Acetoacetate (expressed as CO_2),* μl/100 mg wet wt/2 hr				
	Liver slices			Kidney slices		Liver slices			Kidney slices	
Group	No substrate	Butyrate .017 M	Acetate .08 M	No substrate	Aceto-acetate 300 μl	No substrate	Butyrate	Acetate	No substrate	300 μl aceto-acetate added as substrate
I	180 ± 14	273 ± 15	270 ± 14	550 ± 25	658 ± 30	15 ± 5	78 ± 8	54 ± 6	5 ± 3	68 ± 7
II	153 ± 12	222 ± 20	189 ± 12	540 ± 23	705 ± 32	38 ± 4	96 ± 8	68 ± 6	18 ± 3	145 ± 12
IIa	155 ± 10	229 ± 15	195 ± 11	515 ± 20	690 ± 30	46 ± 5	112 ± 8	76 ± 5	29 ± 5	103 ± 10
III	220 ± 18	329 ± 21	290 ± 19	600 ± 22	909 ± 41	43 ± 5	151 ±13	77 ± 6	20 ± 5	117 ± 11
IV	126 ± 11	150 ± 13	136 ± 12	475 ± 30	600 ± 26	25 ± 6	58 ± 5	21 ± 5	25 ± 6	48 ± 6
V	185 ± 10	270 ± 11	209 ± 9	558 ± 32	704 ± 33	15 ± 4	52 ± 5	25 ± 4	11 ± 4	150 ± 11
VI	210 ± 15	300 ± 18	220 ± 14	—	800 ± 38	17 ± 4	80 ± 7	67 ± 6	—	140 ± 10
VII	180 ± 12	250 ± 15	220 ± 13	—	690 ± 35	12 ± 8	60 ± 5	33 ± 4	—	80 ± 8

* CO_2 in μl produced after decarboxylation of acetoacetate with aniline citrate.

Urine was tested qualitatively for sugar

TABLE II. Blood Sugar, Liver Glycogen, Liver Fat, % of Tissue Weights (Wet Wt) of Different Groups of Rats. 6 rats in each group except Group IIa with 5 rats.

				Wet wt of tissues, g/100 g body wt	
Group	Blood sugar, mg/100 cc	Liver glycogen, g/100 g	Liver fat, g/100 g	Liver	Kidneys
I	84 ± 3	2.45 ± .23	3.4 ± .2	3.5 ± .15	.8 ± .05
II	97 ± 2	1.94 ± .16	5.8 ± .3	5.2 ± .19	.92 ± .03
IIa*	100 ± 3	2.00 ± .12	6.96 ± .25	6.7 ± .29	1.00 ± .06
III	100 ± 3	.29 ± .1	4.8 ± .4	4.1 ± .11	.95 ± .04
IV	90 ± 2	.89 ± .2	5.1 ± .2	5.6 ± .21	.95 ± .05
V	89 ± 2	2.98 ± .17	3.8 ± .3	3.8 ± .16	.82 ± .02
VI	90 ± 3	.95 ± .15	4.6 ± .3	3.9 ± .12	.8 ± .02
VII	—	1.28 ± .16	5.0 ± .23	3.95 ± .2	.9 ± .03

* Rats fed high fat diet for 19 wk.

and ketone bodies weekly.

Results and discussion. Oxygen consumption (Table I) is usually lower in high fat fed rats than in normal animals, and thiouracil feeding further depresses it. Hydrolyzed GCA feeding has been found to correct metabolism of high fat fed rats showing normal oxygen consumption and acetoacetate production. High fat feeding induces higher rate of fatty acid oxidation thereby producing more acetoacetate, which agrees with the results of Tepperman *et al.*(15). Thiouracil exerts a greater influence on high fat diet, and it appears from the results that a high fat diet suppresses the increased metabolic rate in hyperthyroid rats.

Although high fat feeding for a period of 7 weeks shows significant rise in acetoacetate utilization by kidney slices (Table I), when this diet is continued for a further period of 12 weeks, utilization of acetoacetate falls considerably. Production of acetoacetate by liver slices (Group II), however, is higher in high fat fed rats than in controls (Group I). Hydrolyzed GCA feeding has been found to decrease acetoacetate production and to increase its utilization in all groups *i.e.*, Groups V, VI, VII as compared to their respective controls (Table I).

Blood sugar values (Table II) indicate slight hyperglycemic effects in case of high fat diet and hyperthyroidism. Hypertrophy of liver and kidneys was found in these animals. Fat deposition in livers of high fat fed rats, as also observed by French *et al.* (16) is evident from the results in Table II. Liver glycogen in hyperthyroid rats is known to be very low(17) and when high fat fed rats are made hyperthyroid, the same low values are found. Liver glycogen in high fat fed rats is less than the control. Hydrolyzed GCA feeding increases liver glycogen of high fat fed, hyper- and hypothyroid rats. Glycogenic effect of hydrolyzed GCA has previously been reported by us(18). Thiouracil feeding with high fat diets augments hypothyroidism as previously shown by Kennelly *et al.*(19). It reduces ketogenesis and acetoacetate utilization by high fat fed rats (Group IV).

Urine from high fat (Group IIa) and thiouracil fed rats (Group IV) was found to contain traces of sugar and ketone bodies.

The results show the adaptation of animals in respect to fatty acid oxidation when maintained on high fat diet, and other effects of this diet on tissue metabolism.

Summary. High fat feeding increases blood sugar, liver fats and tissue weights (liver and kidneys). It increases acetoacetate production by liver slices and utilization by kidney slices, but this high rate of utilization is not maintained on prolonged feeding (19 weeks). High fat feeding augments hypothyroidism and suppresses hyperthyroidism to some extent. A beneficial effect of hydrolyzed GCA feeding with high fat diets to hyper- and hypothyroid rats is indicated.

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Effect of Mephentermine on Venomotor Tone, Blood Flow and Arterial Pressure in Forearm of Man.* (26179)

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Mephentermine sulfate§ is used effectively in treatment of hypotension(1-3), but there is confusion regarding the means by which the drug restores arterial pressure. A pressor amine may elevate arterial pressure by increasing arteriolar tone, increasing cardiac output or a combination of both(4). Welch *et al.* reported that mephentermine had little effect on peripheral arteriolar resistance in the dog but exerted its effect primarily on myocardial contractility(4). Several authors reported an increase in myocardial contractility after administration of mephentermine without reference to its action on the peripheral arterioles(5,6). Brofman *et al.*(3) found little change in cardiac output in man and dogs and attributed the elevation in arterial pressure to increased peripheral resistance. Aviado(7) reported vasodilation in the dog after intraarterial injection of mephentermine but Borden found vasoconstriction(8).

The effect of a pressor amine on cardiac output will depend not only on response of the myocardium, but also upon the changes induced in venous tone. Venous constriction with a shift of blood from the periphery would increase amount of blood available to the heart and help increase output. Data regarding the effects of mephentermine administration on venous tone in man are not available.

Because of these discrepancies, and since there may be a species difference, it seemed important to study the effect of mephentermine on forearm venous tone and blood flow in man.

Method. Normal male students were studied while lying supine. Room temperature was maintained at 79-81°F and every effort was made to keep the subjects comfortable. They remained in the recumbent position for at least 60 minutes before experiments were begun. Arterial pressure was measured intermittently with a cuff on the right arm. Venous pressure was measured in the antecubital vein of the dependent right arm with a strain gauge. The data were analyzed by the methods described by Fisher (11).

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† Research Fellow of Am. Coll. of Physicians.

‡ Established Investigator of Am. Heart Assn.

§ Wyamine®, Wyeth Labs., Philadelphia, Pa.

Blood flow was measured in the left forearm of 5 subjects by venous occlusion plethysmography(9,10). Temperature of water in the plethysmograph was maintained at 32°C. A cuff was placed distal to the plethysmograph and inflated above systolic pressure during measurements to exclude the circulation in the hand. The congesting cuff was placed close to proximal end of the plethysmograph and optimal collecting pressure, usually about 40 mm Hg, was selected by making preliminary flow measurements. Rates of change of arm volume were recorded with a sensitive pressure transducer, which measured changes in water level in a cylinder attached to the top of the instrument. Blood flow was calculated in ml/min/100 ml of forearm tissue. Flow measurements were made until a steady state was achieved. Control values were then obtained over a 20 minute period by making 4 flow measurements for each 5 minute period. The control value for each experiment is the average of all control measurements. Blood flow measurements after mephentermine were made at the rate of 3 per minute and values reported are averages of flows during the second, third and fourth minute following drug administration. This period includes the peak response but avoids giving undue weight to single observations. Mean blood pressure was calculated by adding one-third of pulse pressure to diastolic pressure. Observations after mephentermine are average values obtained during the second, third and fourth minute after single intravenous injection of 25 mg of the drug. Peripheral resistance was calculated by dividing mean pressure in mm Hg by flow in ml/100 ml for forearm tissue and expressed in arbitrary units.

Venous tone was measured in 5 subjects by the plethysmographic method previously described(12). This method yields information which permits construction of venous pressure-volume curves. These curves express in ml/100 ml of forearm tissue the volume of blood in the forearm veins at any level of transmural pressure between 0 and 30 mm Hg. The final point on the curve, venous volume at 30 mm Hg, is termed arbitrarily *venous distensibility*. A high value represents dilatation or reduced venous tone, a low value represents constriction or increased tone. Since venous pressure-volume characteristics are the same in both arms, the naturally occurring venous volume of the forearm is the volume co-ordinate at the point on the curve which corresponds to venous pressure measured in the arm not in the plethysmograph. This volume co-ordinate is determined by drawing a perpendicular from the point on the pressure axis which corresponds to the level of measured venous pressure. Four control observations were made over a 30 minute period and average values are reported. The recorded forearm venous responses after drug administration represent maximal changes observed after injection of 25 mg.

Results. (Tables I and II) Arterial pressure increased in each experiment. Mean blood pressure increased by an average of 30 mm Hg ($p<0.01$) or 31.8%. Venous pressure increased in each experiment. The average change was 1.7 mm Hg ($p<0.02$) or 13.2%.

TABLE I. Forearm Venous Responses to Mephentermine Administration.

Exp.	Venous pressure, mm Hg Before	Venous pressure, mm Hg After	Venous distensibility, mm/100 ml Before	Venous distensibility, mm/100 ml After	Venous vol, mm/100 ml Before	Venous vol, mm/100 ml After
1	11.0	12.5	4.5	4.1	2.5	2.0
2	19.0	20.0	6.0	4.8	4.8	3.7
3	12.8	16.0	3.8	3.2	2.6	2.3
4	11.5	13.5	4.7	3.7	2.7	2.3
5	15.0	16.0	4.5	3.5	3.0	2.3
Mean	13.9	15.6	4.7	3.9	3.1	2.5
Stand. dev.	3.26	2.90	.81	.62	.96	.67
Mean diff.	+1.7		−.8		−.6	
Stand. error	.41		.14		.14	
Probability	<.02		<.01		<.02	

TABLE II. Effect of Mephentermine on Forearm Blood Flow and Arterial Pressure.

Exp.	Forearm blood flow, ml/100 ml		Blood pressure, mm Hg		Resistance, arbitrary units*	
	Before	After†	Before	After	Before	After
1	4.0	3.2	98	115	24.5	35.9
2	2.0	1.5	86	104	43.0	69.3
3	2.3	1.8	95	130	41.3	72.2
4	4.3	1.7	95	131	22.0	77.0
5	4.6	2.2	98	143	21.3	65.0
Mean	3.4	2.1	94	125	30.4	63.9
Stand. dev.	1.2	.7	4.9	15.2	10.8	16.2
Mean diff.		−1.4		+30.0		+33.5
Stand. error		.47		5.47		7.46
Probability		<.05		<.01		<.02

* Ratio of mean arterial pressure to blood flow.
† Avg values for 2nd, 3rd, and 4th min. after drug admin.

Venous distensibility decreased in each of the 8 experiments. Average decrease in distensibility was 0.8 ml/100 ml of forearm tissue ($p<0.01$), a change of 22.1%. The naturally occurring venous volume decreased from an average of 3.1 ml/100 ml to 2.5 ml ($p<0.02$), a decrease of 18.5%. Even though the pressure distending the veins increased, venous constriction was sufficient to shift blood from the forearm. Blood flow for the second, third and fourth minutes after drug administration decreased in each of the 5 experiments. Decrease in flow averaged 26.6% or 1.4 ml/100 ml of forearm tissue ($p<0.05$). Peak reduction in flow occurred within 1 to 2 minutes and returned to near control levels within about 10 minutes.

Calculated peripheral resistance increased from an average of 30 units during control periods to 64 units after mephentermine administration ($p<0.02$).

Discussion. These results show that forearm blood flow decreases in normal men after intravenous administration of mephentermine. Rapid single injections of the drug were used for this study but similar results were obtained in one subject when the experiment was repeated with a 5 minute intravenous infusion of mephentermine. These observations are in agreement with Borden (8) and Brofman(3). They do not indicate, however, that the same response should or would be expected in the subject with hypotension. In many forms of acute hypotension, there may be loss of venomotor tone and pooling of blood in the periphery(13). This reduces venous return and leads to a fall in cardiac output. Mephentermine produces peripheral venous constriction with a shift of blood from the periphery as these experiments show. Since it also increases contractility(4, 5,6), it would be expected to restore cardiac output. This increase in total blood flow could restore arterial pressure without an associated increase in arterial resistance.

We measured cardiac output in 14 normotensive and hypotensive dogs and found that mephentermine caused it to increase in each instance. In 5 dogs, however, arterial pressure increased appreciably immediately after the drug before cardiac output changed. This indicates that mephentermine produced an increase in arteriolar tone initially. The later effect was increased resistance as well as increased arterial pressure and total blood flow.

The changes in forearm venous tone and blood flow after mephentermine could be due in part to an effect on the central nervous system, a direct action on the vessel or reflex neural adjustment secondary to an alteration in cardiac output. Swaine(15) reported evidence that mephentermine increases cardiac output primarily by releasing catechol amines. He suggested that this effect was limited to the myocardium and did not influence response of the peripheral vessels. If mephentermine exerts its effect on the myocardium by releasing catechol amines, it might act in the same way on the peripheral vessels. Such a hypothesis would be plausible since norepinephrine has been reported to produce venous constriction(12) and to reduce blood flow in the extremity(16).

Summary. Forearm blood flow and venomotor responses to intravenous injection of mephentermine were studied in normal men. In each experiment blood flow decreased while arterial pressure increased. Venous pressure increased while forearm venous distensibility and venous volume decreased in each subject. In each case blood shifted from the forearm. These experiments indicate that mephentermine administration increases both peripheral resistance and venous tone in man.

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A Species Comparison of Serum Proteins and Enzymes by Starch Gel Electrophoresis.* (26180)

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Employment of starch gel electrophoresis as a means of protein separation has found wide application. Serum proteins(1), hormones(2), serum(3,4) and tissue(5) enzymes have been characterized by this technic. Although several recent investigations have been concerned with the enzymes of human serum(3,4), little information is available on the enzymes of laboratory animals. Protein migration in a number of animals(6-11) and esterase migration in starch block in several species(12) have been published. In the present study the occurrence and distribution of esterase, cholinesterase, aminopeptidase, cytochrome oxidase, beta-glucuronidase, acid and alkaline phosphatase and ceruloplasmin oxidase in the sera of man and 6 species of experimental animals are compared. Succinic, lactic, beta-hydroxybutyric and glutamic dehydrogenases were also studied in human serum. To provide a broader basis of comparison and for orientation, serum protein patterns are also included.

Materials and methods. Species studied and number of individuals in each follow: Man (Caucasian) 30, monkey (Cynomologus from Philippines) 3, dog (mongrel) 3, rabbit (New Zealand White) 8, guinea pig (hybrid) 8, rat (Sprague-Dawley) 8, mouse (Webster strain of Swiss) 10. All subjects were adult males.

Starch gel electrophoresis was carried out under conditions described by Smithies(1). Specimens were absorbed on filter paper and inserted in a slot 10 cm from the cathode in parallel with a specimen of human serum. Following electrophoresis, the gels were sliced in half longitudinally. One half was stained for protein with Amido-black 10 B and enzymatic activity was determined in the other (4). Gels were photographed and schematic representations prepared. Differences in mi-

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gration were compensated by differential enlargement employing the leading edge of the human albumin band as an index.

Technic of Stains: *Aminopeptidase.* 50 ml Tris-maleate buffer, 0.1 M, pH 6.8; 20 mg L-leucyl-beta-naphthylamide (Dejac Laboratories; this dissolves with difficulty but should not be heated); 30 mg Black K salt; incubate 3 hours at 37°C with occasional gentle shaking.

Beta-glucuronidase. 30 ml acetate buffer, 0.1 M, pH 5.2; 15 mg 8-hydroxy-quinoline glucuronide (dissolves with difficulty but can be safely heated to the temperature of the paraffin oven, 56°C); 20 mg Blue RR salt; incubate 16 hours at 37°C.

Cholinesterase. 50 ml Tris-maleate buffer, 0.1 M, pH 6.4; 20 mg 6-bromo-2-naphthylcarbonaphthoxy choline iodide (Dejac Laboratories); 25 mg Blue B salt; incubate 1 hour with occasional gentle shaking at room temperature.

Esterase. 50 ml Tris-maleate buffer, 0.1 M, pH 6.4; 2 ml 1% alpha-naphthyl acetate in 50% acetone; 25 mg Blue B salt; incubate 1 hour with occasional gentle shaking at room temperature.

Alkaline phosphatase. 50 ml Tris (not Tris-maleate) buffer, 0.1 M pH 8.8; 50 mg sodium alpha-naphthyl phosphate; 50 mg Blue RR salt; 5 drops of 10% aqueous solution magnesium chloride; incubate 16 hours at room temperature or 6 hours at 37°C.

Acid phosphatase. 50 ml acetate buffer, 0.1 M, pH 5.0; 50 mg sodium alpha-naphthyl phosphate; 50 mg Red 3 GS salt; 5 drops of 10% solution of magnesium chloride; 5 drops of 10% solution manganese chloride; 1 g sodium chloride; 0.25 g polyvinylpyrollidone; incubate 24 hours at room temperature (22°C).

Cytochrome oxidase. 50 ml phosphate buffer, 0.1 M, pH 7.5; 2 ml 1% alpha naphthol in 40% ethyl alcohol; 2 ml aqueous 1% dimethylparaphenylenediamine; incubate 2 hours with occasional gentle shaking at room temperature.

Succinic dehydrogenase. 2.5 ml phosphate buffer, 0.2 M, pH 7.6; 2.5 ml 0.2 M sodium succinate; 5 ml aqueous solution of Nitro B T, 1 mg per ml; 0.25 ml phenazine methosulfate solution, 5 mg per ml; incubate 1 hour at 37°C.

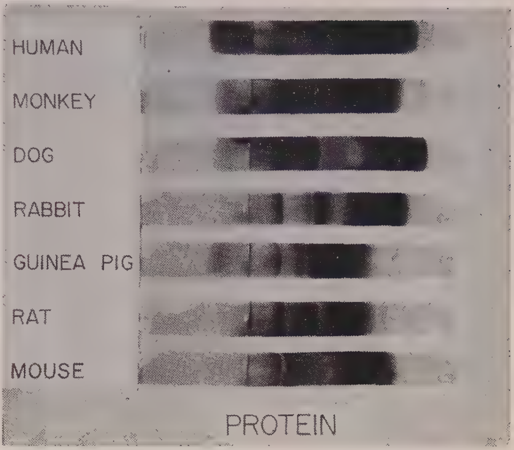


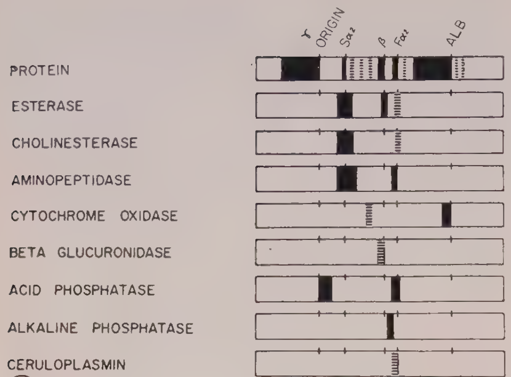
FIG. 1. Comparison of starch gels of 7 mammalian species stained for protein. (Not differentially enlarged.)

Lactic dehydrogenase. 1.0 ml of 1.0 M aqueous solution of sodium DL-lactate; 1.0 ml of 0.1 M aqueous solution of DPN (prepared from a 1.0 M solution kept at a slightly acid pH in the deep freeze); 1.0 ml of 0.1 M aqueous solution of sodium cyanide; 1.0 ml of 0.05 M aqueous solution of magnesium chloride; 2.5 ml 0.1 M phosphate buffer, pH 6.8; 2.5 ml aqueous solution Nitro B T, 1 mg per ml; distilled water to make a total of 10 ml; incubate 1 hour at 37°C.

Beta-hydroxybutyric dehydrogenase. The conditions for this enzyme were identical with those for lactic dehydrogenase, except that 1.0 ml of 1.0 M aqueous solution of DL-beta-hydroxybutyric acid was used as substrate.

Glutamic dehydrogenase. The technic was identical with that for the above 2 enzymes, except that 1.0 ml of 1.0 M aqueous sodium L-glutamate monohydrate was used as substrate.

Results. Serum protein distribution of the various species is shown in Fig. 1. In all species, reference is made to the nomenclature employed by Smithies for man(1). It may not be inferred that proteins which have similar mobilities in starch gels are identical. The protein patterns of all experimental animals were less complex than those of man. Pre-albumin was observed in only 3 species, human, monkey and mouse. Mouse and rat



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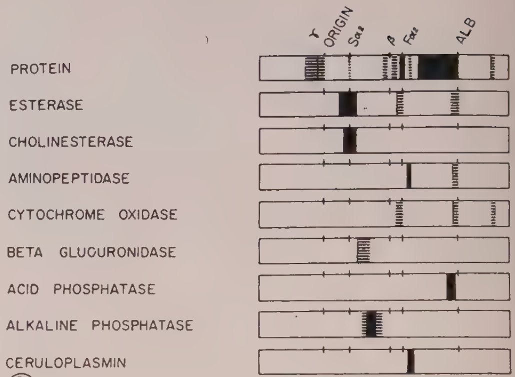

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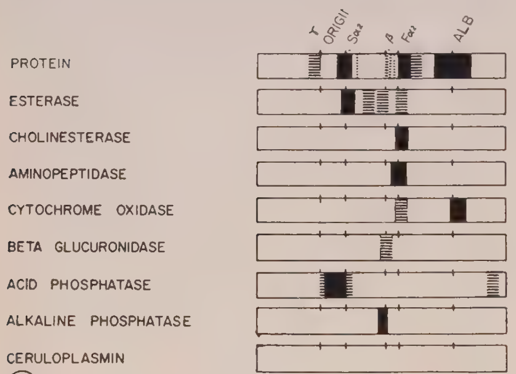

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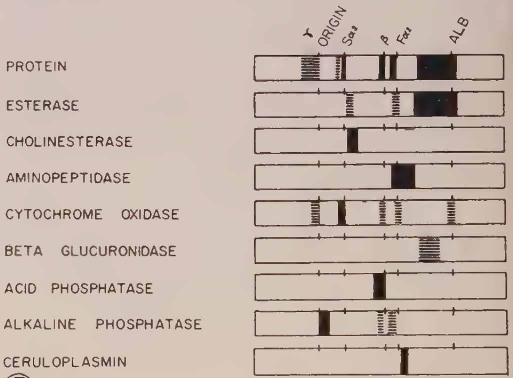

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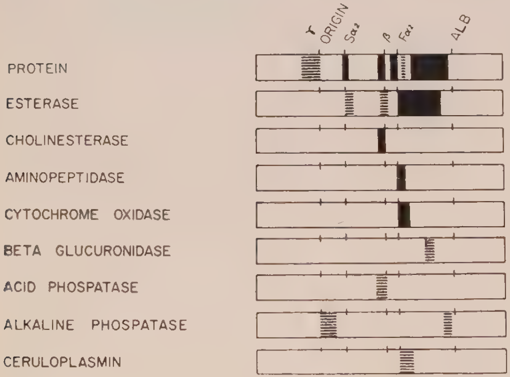

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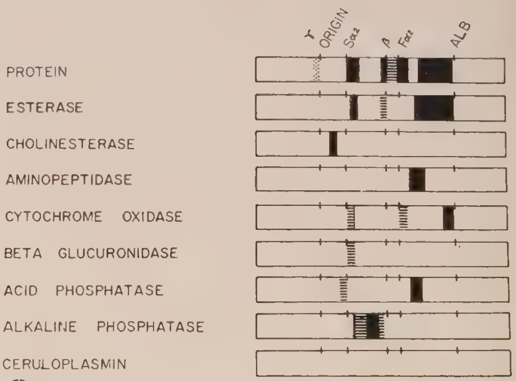

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FIG. 2. Enzyme distribution in human. Dashed bands represent less activity than solid.
FIG. 3. Enzyme distribution in the monkey.
FIG. 4. Enzyme distribution in the dog.
FIG. 5. Enzyme distribution in the rabbit.
FIG. 6. Enzyme distribution in the guinea pig.
FIG. 7. Enzyme distribution in the rat.

serum samples exhibited very low gamma-globulin levels.

Fig. 2-8 illustrate occurrence and distribution of enzymes in each species. The full complement of enzymes was found in all species except the dog and the rat in which ceruloplasmin oxidase could not be demonstrated. Greatest esterase activity, as judged by width of bands and intensity of staining, was found in the mouse. Cholinesterase was observed in

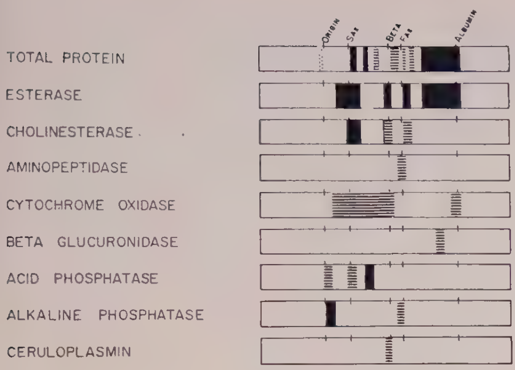

FIG. 8. Enzyme distribution in the mouse.

fractions with low mobility in all species except the dog. Cytochrome oxidase exhibited the greatest divergence with respect to number of bands, varying from one in the guinea pig to 5 in the rabbit. Beta-glucuronidase activity was found in only one band in all species. It, along with aminopeptidase and ceruloplasmin, had the most uniform migration. Although there was some variability in intensity of the various zones after both protein and enzyme stains among various members of the same species, the overall pattern for that species was constant.

Summary. The occurrence and distribution of serum enzymes and proteins in 7 mammalian species have been studied by means of starch gel electrophoresis and appropriate staining technics. Species differences were observed with respect to the presence, number, mobility, and concentration of components exhibiting various enzymatic activities. Similar observations were also made with respect to bands staining for protein.

The technical assistance of Carl Howell and John A. Cogswell is gratefully acknowledged.

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Comparison of "Meal Eating" and "Nibbling" on Severity of Alloxan Diabetes.* (26181)

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That rate of ingestion of diet plays a significant role in regulation of over-all body metabolism is suggested by the following data: animals fed full-spaced meals ("meal eaters") demonstrate (a) increased quantities of body fat, (b) decreased body protein, (c) altered thyroid activity and (d) changed tissue enzymatic activity, when compared to animals eating the same diet in frequent small feedings ("nibblers")(1). To determine whether manner of eating plays a role in severity of diabetes, experiments were undertaken dealing with relationship of eating habits to glycosuria and nitrogen excretion in alloxanized rats. Results indicate that rate of ingestion of diet does influence excretion of both substances in presence of experimental alloxan diabetes.

Methods. Male Holtzman rats, received when approximately 100 g in weight, were placed on a 50% carbohydrate diet *ad libitum*, and when about 130 g were given 50

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TABLE I. Effect of Rate of Ingestion of Diet on Urinary Glucose and NPN Excretion in Alloxan Diabetic Rats.

		Cumulative excretion*					
		Glucose			NPN		
Rat No.	Diet and days of observation	*Ad libitum*	Force fed	% increase†	*Ad libitum*	Force fed	% increase†
Series 1							
1	50% CHO 8 days	12224	18238	49	1824	2312	27
2	*idem*	1605	4613	187	1448	1776	23
3	”	287	548	91	1928	1968	2
5	”	667	2144	221	1784	2072	16
6	”	451	1523	238	2120	2496	18
10	”	171	1082	536	1608	2424	51
	Mean increase in excretion, ± S.E.			220 ± 70			23 ± 6.6
Series 2							
10	50% CHO 7 days	373	871	134	1610	2198	37
5	*idem*	471	1322	181	2002	2219	11
11	”	852	1417	66	1603	2198	37
15	”	18273	20645	13	1960	2149	10
	Mean increase in excretion, ± S.E.			99 ± 37			24 ± 7.6
Series 3							
10	71% CHO 6 days	275	1139	314			
5	*idem*	141	1075	662			
11	”	258	974	278			
15	”	11297	15348	36			
	Mean increase in excretion, ± S.E.			323 ± 129			

* The excretions are given in mg for total No. of days; food ingested the same for both periods.

† Excretion during *ad libitum* period = 100%.

mg/100 g body weight of alloxan (“sub-diabetogenic” dose) at pH 4.0 subcutaneously (*2*). One week later, animals were started and continued, for duration of experimental periods of observation, on subcutaneous injections of 2 U protamine Zn insulin‡ daily. Experiments started 4-8 weeks after alloxanization.

At start of periods of observation, rats were put into individual metabolism cages, their *ad libitum* food intake measured daily and their 24 hour urines collected in citric acid under toluene for 6, 7 or 8 days. Animals were then adapted to a forced feeding regimen with the same diet twice daily; by the end of 2 weeks, it was possible to force feed each day the same quantity of food each animal had previously consumed so that every rat was his own control (“pair-fed” against itself). Urines were collected daily throughout. Four rats were put through the *ad libitum*-force feeding cycle once, 4 rats were cycled twice and 2 rats cycled 3 times. Two of the cycles were accomplished with a moderate (50%) carbohydrate diet and one with a high (71%) carbohydrate diet. Preparation and composition of diets were described previously(3,4). Urinary glucose determinations were accomplished by the method of Nelson(5), urinary NPN evaluated according to Conway(6). The results are given as total urinary excretions during number of days of study.

Results and discussion. When animals were force fed twice daily the same quantities of diet they had eaten previously *ad libitum*, excretions of glucose and NPN were significantly greater than excretions measured under nibbling conditions. These findings were true not only for the total period but for each comparative day. The increase in glycosuria varied between 13% and 662%, averaging 215%. NPN excretions increased by an average of 23% (Table I). A greater loss of glucose and nitrogen occurred during the force-feeding period, irrespective of whether diabetes was “poorly” or “well” controlled during nibbling period.

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‡ Generously supplied by Dr. W. R. Kirtley, Eli Lilly Co.

The increased glycosuria observed with force feeding could have resulted from either greater postprandial hyperglycemia attendant on this manner of feeding or from a relatively more marked insulin deficiency. It is conceivable that, with the decreased rate of protein synthesis that accompanies the meal eating regimen, synthesis of the protein hormone insulin might be affected likewise. The effects of forced feeding on nitrogen metabolism might be attributed to (a) a rate-limited ability of the body to handle ingested protein or (b) increased gluconeogenesis. It has been observed(1) that "pair-feeding" rats in a limited time period (giving the amount of diet consumed by other animals over a 24 hour period) results in a decreased ability to store body protein. The data of Wu and Wu (7) in the human suggest that this finding results from a limitation in quantity of protein the organism can handle in a given time period. Also, a possible cause for the increased NPN excretion seen with forced feeding is accelerated gluconeogenesis, secondary to the increased glycosuria. However, both our data and those of Wu and Wu favor rate-limitation as the cause for the greater nitrogen excretion that accompanies forced feeding.

Animals were recycled through the two feeding patterns to exclude the exacerbations and remissions that characterize alloxan diabetes(8) as being responsible for our findings. Constant insulin dosage was suboptimal in order to compare glycosuria under the conditions of both *ad libitum* and forced feeding.

The implications of our findings with respect to man appear obvious. Increased frequency of feedings is now used in therapy of complications of diabetes. During treatment of diabetic acidosis, glucose when given is administered intravenously in small constant dosage. When "brittle" diabetics are in need of control or a diabetic patient is faced with complications making regulation difficult, feedings every 6 hours have been given(9,10). Our results suggest that in therapy of human diabetics, the use of more frequent meals might lead to better control and to reduction in insulin dosage needed.

Summary. The food intake and urinary excretion of glucose and NPN were measured daily in alloxan diabetic rats under the conditions of free access to food. The animals were then adapted to force feeding and fed in this manner twice daily the same quantity of diet they had consumed *ad libitum*. Insulin dosage was kept constant throughout. Under conditions of forced feeding, glycosuria increased by 215% and NPN excretion by 23%. These findings were reproducible when animals were recycled 2 or 3 times, demonstrating that eating patterns, not ebb and flow of severity of diabetes, were responsible for the findings.

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Androgenic Activity in the Interscapular Brown Adipose Tissue of the Male Hibernating Bat (*Myotis lucifugus*).* (26182)

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In most mammals the primary and accessory sex gland cycles occur at the same time and the interstitial tissue of the testis is thought to be the source of the androgen promoting this synchronization. In *Corynorhinus* and *Myotis* (see Pearson *et al.*(1) and Miller(2)) there is an apparent dysynchrony between primary and secondary sex glands. In these mammals, spermatogenic activity and interstitial cell and testis size decline rapidly at a time when the accessory glands are undergoing hypertrophy. Accessory glands remain enlarged during the season when testes are shrunken and interstitial cells are small and appear nonsecretory. Pearson *et al.*(1) castrated such animals and found that accessory glands were smaller than noncastrated controls. Nevertheless, histochemical tests to show the presence of androgens and other ketosteroids in the testis of such operated bats were negative. Similar results have been obtained by one of us (P.H.K. unpublished data) in studies on the bat *Myotis lucifugus*. Such information suggests the possibility of the presence of androgen elsewhere in these bats.

Evidence that brown fat of hibernating mammals may contain steroids is offered by Sweet and Hoskins(3) who found, by biological assay, that an alcoholic extract of the "hibernating glands" (interscapular brown fat) of the woodchuck contained the equivalent of 1 International Unit of androgen in 50 g of tissue. These authors suggested that rapid utilization of this tissue following emergence from hibernation, which is coincident with onset of the breeding season, may reflect the part played by the hibernating gland in the sex cycle.

The purpose of our study has been to examine the possibility that there is androgenic activity in the interscapular brown adipose tissue of the male hibernating Vespertilionid bat *Myotis lucifugus*. An effort has been made to determine degree of androgenic activity in this tissue by comparison of seminal vesicle gland growth in weanling rats given various concentrations of testosterone to that of weanling rats receiving a nonsaponifiable fraction of the interscapular brown adipose tissue (NFBF). Results to be reported suggest that interscapular brown adipose tissue provides a notable androgenic stimulus to the target organ (seminal vesicle) in bioassay.

Materials and methods. Interscapular brown adipose tissue was taken from hibernating adult male bats (*Myotis lucifugus*). The bats for each individual experiment were obtained not more than 3 days prior to experiment from various caves in Pendelton Co., West Va. In the laboratory the bats were kept torpid (50°F) until their sacrifice by ether. During this study 3 separate preparations were analyzed for androgenic activity. In a typical isolation, the interscapular brown fat from 30 bats was removed, weighed (4.39 g) and saponified with 50 ml of 20% KOH in 85% ethanol under a stream of nitrogen for 2 hours. The nonsaponifiable fraction was dissolved in diethyl ether, thoroughly washed with distilled water and the mixture taken to dryness, *in vacuo*. The nonsaponifiable residue (*Ca.* 86.0 mg) was dissolved in 8 ml of 20% Tween-80 (polyoxyethylene sorbitan monooleate, Atlas Powder Co.). In one experiment, the nonsaponifiable fraction from 4.94 g of liver (21 bats), 34 mg of adrenal glands (30 bats) and 803 mg of testis plus epididymus (30 bats) was prepared as described above.

Biological assays. Response of the seminal vesicles of weanling male rats (avg initial wt., 52.5 g) of the Holtzman Strain to the NFBF from bats was compared to that resulting from various levels of testosterone (Merrell).

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TABLE I. Effect of Testosterone and NFBF on Seminal Vesicles of Intact Weanling Male Rats.

Group*	Mean relative sem. vesicle wt (mg of tissue/g body wt) Exp. I	Exp. II	Exp. III
Tween-80	.30 ± .02†	.24 ± .01†	.23 ± .01†
50 μg testosterone	.38 ± .02	.37 ± .02	.38 ± .03
150 " "	.42 ± .02	.49 ± .03	.64 ± .01
300 " "	.53 ± .01	.59 ± .02	.81 ± .02
NFBF	.48 ± .03	.44 ± .02	.63 ± .02

* Each group in the assay contained 10-11 weanling rats except the NFBF groups which consisted of 3, 3 and 6 animals for Exp. I, II and III respectively.

† Stand. error of mean, $\sigma_M = \frac{\sigma}{\sqrt{N-1}}$.

Animals were divided into 5 groups according to the inoculum received (Table I) and all animals for each experiment were injected concurrently. Daily subcutaneous injections of 0.25 ml were administered on the dorsum of the trunk for 4 days. Testosterone (50 μg, 150 μg and 300 μg standard doses), NFBF and nonsaponifiable matter from liver, adrenal glands and testis plus epididymus were administered in 20%-Tween-80. The control animals received only the solvent. Animals were sacrificed on the fifth day, and seminal vesicles removed and weighed on a torsion balance to the nearest tenth of a milligram. Seminal vesicles were not freed of their contents before weighing. Sample tissues from the seminal vesicles of all animals were fixed in Zenker's fluid, embedded in paraffin, sectioned at 5 μ and stained with Harris's hematoxylin and eosin for microscopic examination.

Results. The response of the seminal vesicle glands of weanling rats to standard doses of testosterone and NFBF for each experiment are presented in Table I. In each experiment NFBF elicited an increase in mean weight of the seminal vesicles of weanling rats equivalent to that produced by 4 daily doses of 150 μg of testosterone. On the basis of mean weight of the seminal vesicles, androgenic activity of the total NFBF (average of experiments I, II and III) may be calculated to be the equivalent of 676 μg testosterone per g. This is a remarkable amount of androgenic activity and vastly exceeds that of bull testis, the richest tissue source heretofore known(4). Control tissue preparations revealed the absence of androgenic material from the liver and small but insignificant quantities of androgenic activity from the adrenal and testis plus epididymus tissues.

Histological change of the epithelium of the seminal vesicles is much less dramatic and allows at best only a subjective evaluation of the androgenic activity of the NFBF. Epithelium of the seminal vesicles of all groups varied from low cuboidal to tall columnar. Nuclei were often basal and degree of granulation of the supranuclear zone of cytoplasm appeared to be in direct proportion to amount of androgen received. Control animals exhibited a greater percentage of low columnar or cuboidal epithelial cells and their supranuclear cytoplasm was often agranular; whereas, the epithelium of animals receiving 4 daily doses of 300 μg of testosterone each was more often tall columnar with the supranuclear cytoplasm frequently granular. The remaining groups (given NFBF and smaller doses of testosterone) demonstrated intermediate states (Fig. 1) in epithelial activity. The stroma was nearly alike in all groups.

Discussion. Information available on the presence of steroids and sterols in the brown adipose tissue of mammals is scarce and contradictory. Clément(5), employing biochemical methods, noted only faint traces of sterols in the interscapular brown fat of rats. Cramer(6) reported positive results in Liebermann-Burchard tests for sterols in warm chloroform suspensions of perirenal brown fat of rats and Tonitti(7) found considerable cholesterol in the interscapular fat bodies of mice. On the other hand Baker, Ingle and Li(8) and Fawcett(9), using the Schultz reaction for cholesterol, were unable to detect sterols in the brown adipose tissue of rats and the Liebermann-Burchard reaction on chloroform extracts of rat brown fat was likewise negative. Fawcett(9) found that birefringence tests for steroids on frozen sections of rat brown fat gave negative results; however, Baker, *et al.*(8) observed abundant birefringent material which they felt suggested the presence of cholesterol or its esters. Fawcett

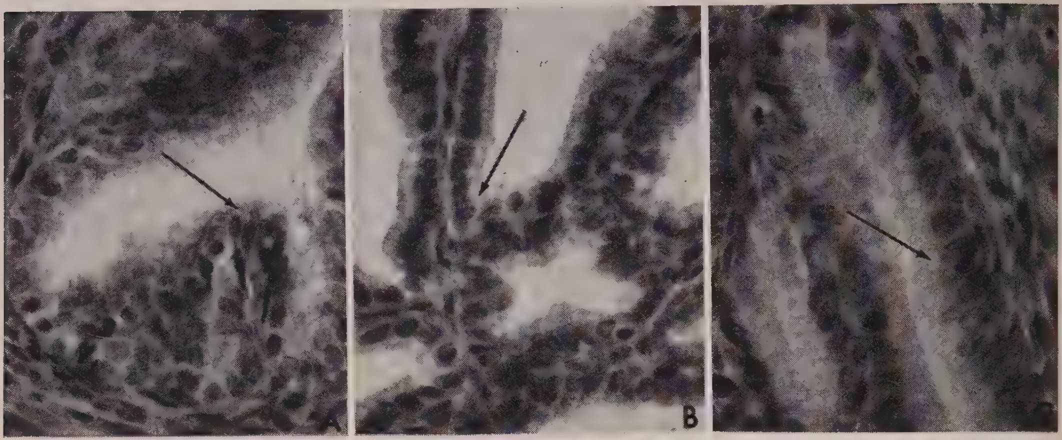

FIG. 1. Photomicrographs of a representative area from the seminal vesicle of: A. Control, B. NFBF and C. testosterone (300 μg/day). Note the difference in amount of supranuclear cytoplasm (at tip of arrow). × 366.

(9) found that the Ashbel and Seligman reaction, which is capable of revealing ketonic steroids *in vitro* and in frozen sections of endocrine glands, gave a negative reaction when applied to brown fat.

As pointed out by Remillard(10) most results seem to indicate the absence of steroid in the brown fat tissues of non-hibernating mammals whereas the few observations available on the brown fat tissue of hibernating mammals suggest that the contrary condition exists.

Remillard(10), using chemical technics, observed small amounts of total cholesterol in the interscapular brown adipose tissue of the female bat (*Myotis lucifugus*). The greatest concentration of total cholesterol occurs in the summer months (highest in August) and minimum concentration is reached in December. Fluctuation in cholesterol levels during summer months seem to accompany drops or rises in water content of the brown fat. Early in hibernation there seems to be a decrease in concentration of total cholesterol, followed by a rise in total cholesterol level during the second half of the period of hibernation. It has been suggested that this later increase may correspond to a preparation for awakening which is accompanied by a higher rate of metabolism and an intensified sexual activity(10). Using histochemical technics, Remillard(10) was able to demonstrate only esters of sterol (principally cholesterol esters).

The high level of androgen in the interscapular brown fat of the bat reported here suggests that the interscapular brown adipose tissue supports accessory gland hypertrophy during testicular involution. Studies are in progress to determine the chemical identity of this androgenic substance[†] and to ascertain whether or not it is actually synthesized or merely stored in the interscapular gland. Further, it will be of interest to evaluate androgenic potency of the brown fat of the bat during the season of testicular hypertrophy.

Summary. *Myotis lucifugus*, simultaneously exhibits enlarged accessory sex glands and atrophied testis during hibernation. Evidence is presented for presence of an unidentified androgenic substance in the interscapular brown fat obtained from the bat during the hibernation season. The active substance isolated from the nonsaponifiable matter of one gram of brown fat elicits a growth response (rat seminal vesicle assay) equivalent to that produced by 676 μg of testosterone. The presence of an androgenic factor in

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[†] Preliminary gas chromatography of the NFBF revealed the presence of a compound that possesses the retention time characteristic of C-19 steroids. The retention time, however, is unlike that of any steroid previously reported(11), nor is it identical to that of testosterone or androsterone which were analyzed as reference compounds. We are indebted for these analyses to Dr. Charles C. Sweeley, Dept. of Biochemistry and Nutrition, Graduate School of Public Health.

brown fat suggests a unique role for this tissue in the sexual cycle of the bat.

We wish to acknowledge the valuable assistance of Messrs. Charles R. Cook and Robert Kolek.

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Effect of Histamine Liberators on Urinary Excretion of 5-Hydroxyindolacetic Acid.[*][†] (26183)

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Bhattacharya and Lewis showed(1) that, in addition to histamine, serotonin is released from rat tissues perfused with Compound 48/80, a potent histamine liberator. Parratt & West(2,3) demonstrated that the anaphylactoid reaction induced in rats by dextran or egg white is chiefly mediated by release of serotonin. Further to these observations, a study was undertaken to evaluate urinary excretion of 5-hydroxyindolacetic acid (5-HIAA) in rats receiving various histamine liberators. The results reported here show that the output of the serotonin metabolite is unchanged in animals treated with histamine liberators, even after repeated administration. When rats were made resistant to anaphylactoid edema by prolonged treatment with Compound 48/80, they still excreted as much 5-HIAA after injection of reserpine as did the untreated controls.

Materials and methods. Female rats of the Holtzman strain, weighing between 120 and 150 g, were used in these experiments. Twenty-four-hour urine specimens were collected in metabolic cages. During that time, animals were not fed but were adequately hydrated by gastric instillation of 2 ml of tap water 4 times daily. To keep urinary pH sufficiently low, a small amount of acetic acid was added to the flasks. Average urinary output was 10 ml and half of this volume was used for determination of 5-HIAA, following the method of Pierce(4).

In the first experiment, histamine liberators were injected once, at doses indicated in Table I, immediately before animals were caged. *In the second experiment,* Compound 48/80 was administered during 8 days in increasing doses ranging from 200 γ to 1 mg, as reported elsewhere(5), in order to desensitize animals to anaphylactoid edema. In fact, these rats manifested resistance to dextran (6 mg i.p.) and polymyxin B (1 mg s.c.). On the ninth day, they were challenged with 0.5 mg of reserpine, subcutaneously, and placed in metabolic cages for collection of urine.

Results. The values for urinary 5-HIAA are expressed in the Tables as μg/ml. Esti-

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TABLE I. Urinary Excretion of 5-HIAA in Rats Treated with Histamine Liberators.*

Groups of 6 rats	Dose, mg/day	Urinary 5-HIAA, μg/ml	"P" value relative to controls	Urine output, ml/24 hr
Controls	—	5.2 ± .21†	—	12.2 ± .45†
Compound 48/80	.25 × 2 s.c.	5.0 ± .51	>.5	12.3 ± 1.14
Polymyxin B	1. s.c.	5.3 ± .71	>.5	10.7 ± .97
Dextran	12. i.p.	5.8 ± .09	>.05	10.6 ± .87
Serotonin	.5 × 2 s.c.	19.1 ± 1.64		11.0 ± .84

* Animals were edematous during 6 hr.

† Mean ± S.E.

mation of 24 hour values is obtained by multiplying these figures by total volume of urine excreted. Table I reveals that there are no significant differences between control rats and those treated with histamine liberators. Prolonged treatment with Compound 48/80 did not further modify excretion of 5-HIAA (Table II). In addition, it can be seen that animals so treated, although resistant to anaphylactoid reaction, still excreted as much 5-HIAA after reserpine injection as did non-pretreated rats.

Discussion. The ineffectiveness of histamine liberators to increase urinary excretion of 5-HIAA does not substantiate the claim that such liberators release serotonin. Failure to obtain this evidence may be ascribed to the fact that the effects of histamine liberators in living animals differ from those observed in perfusion experiments reported by Bhattacharya and Lewis(1). The optimal dose tolerated *in vivo* is perhaps inadequate to release sufficient tissue serotonin and raise urinary 5-HIAA. The studies of Lecomte seem quite relevant in this respect. It was observed that urinary 5-HIAA augments during anaphylaxis only in those animals which exhibit a severe state of shock(6,7).

TABLE II. Effect of Reserpine upon Urinary Excretion of 5-HIAA in Rats after Prolonged Treatment with Compound 48/80.

Groups of 12 rats	Urinary 5-HIAA, μg/ml	"P" value	Urine output, ml/24 hr
Controls	4.8 ± .32‡	.2	10.8 ± .43‡
Compound 48/80*	5.4 ± .38		12. ± .59
Reserpine†	9.4 ± .39	.4	7.75 ± .34
Compound 48/80* + reserpine† (0.5 mg s.c.)	10.6 ± .57		9.6 ± 1.20

* Inj. s.c. during 7 days in increasing doses of 0.1 to 0.5 mg, twice daily.

† 0.5 mg inj. once s.c. following treatment with Compound 48/80.

‡ Mean ± S.E.

The present experiments also provide no support to the hypothesis that serotonin is the chief mediator of the anaphylactoid reaction. This is evidenced by the fact that rats made resistant to anaphylactoid edema react normally to the depleting action of reserpine on tissue serotonin. It may be assumed that Compound 48/80 and reserpine have 2 different sites of depleting action in rats but, should this be the case, the role of serotonin in the genesis of the cutaneous edema remains unexplained. In addition, one normally expects that serotonin liberation that occurs after reserpine treatment would give rise to anaphylactoid edema, and this is not the case. Reserpine, on the other hand, when given in sufficiently high doses, *i.e.,* 10 mg/kg, was shown by Parratt and West to desensitize rats to edema-producing agents(2). Following this treatment, animals become lethargic and do not eat or drink. However, the concurrent treatment with a central stimulant, such as methylphenidate, and adequate hydration by stomach tube will restore their sensitivity to dextran (unpublished).

It was demonstrated previously that susceptibility of rats to anaphylactoid reaction is closely related to the number and integrity of cutaneous mast cells(8). Tissue mast cells have been found unchanged in reserpinized animals(9,10), while 5-HIAA increases in the urine. This picture is the reverse of that seen during anaphylactoid reaction in rats where mast cells are degranulated but there is no concomitant increase of urinary 5-HIAA.

Summary. Urinary excretion of 5-hydroxyindolacetic acid (5-HIAA) has been evaluated in rats treated with various histamine liberators. Excretion of 5-HIAA was

unchanged, even when the liberator was administered repeatedly. When rats were given Compound 48/80 in increasing dosage during 8 days, they were entirely resistant to edema normally produced by other liberators. These animals, however, excreted as much 5-HIAA after injection of reserpine as did the untreated controls. These observations do not militate in favor of a serotonin mediation in development of anaphylactoid edema in rats.

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Metabolic Effects of *m*-Dinitrobenzene in *Aspergillus niger*.* (26184)

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m-Dinitrobenzene (DNB) is a potent growth inhibitor for molds; concentrations as low as 2.5 x 10^{-5} M produce complete stasis of *Aspergillus niger* growth. Casein hydrolyzates and amino acid mixtures reversed inhibition but it was not possible to establish mechanism of action for these phenomena(1). The present communication reports metabolic observations which are consistent with the proposal that DNB inhibits mold growth by indirectly depressing amino acid synthesis during early growth phase.

Methods and results. Culturing procedures for *A. niger* (Mulder strain)(2) and growth rate methods were as described previously (1). DNB solutions were prepared fresh weekly since activity is gradually lost on standing in stoppered glass containers for longer periods. Although growth inhibitory properties of DNB were overcome by various amino acid mixtures(1), leucine and proline were the only amino acids which, when omitted individually from such mixtures, prevented this reversal. These 2 compounds were, in fact, nearly as effective as the whole mixture of 19 amino acids when substituted on an equal weight basis. Hydroxyproline was without effect, but aspartic and glutamic acids were partially effective substitutes for proline. These results are in accord with known metabolic interconversions of amino acids. It is significant that these 4 effective amino acids constitute 50% of the casein molecule.

In addition to amino acids, certain intermediates of carbohydrate metabolism were also effective in reversing DNB growth inhibition (Table I) and these effects were most pronounced between 5th and 6th day of growth. (Decrease in weight of controls is due to autolytic changes after 5th day). An equivalent amount of a mixture of citric, α-ketoglutaric, succinic and oxalacetic acids

TABLE I. Reversal of DNB Growth Inhibition in *A. niger*.*

Additions†	Incubation time (days) 5	6	7
None	836	751	667
1.0 μmole DNB	17	23	59
Idem + leucine + proline‡	194	342	479
" + citric acid	240	811	837
" + succinic acid	39	457	727

* mg dry wt of mycelium (50 ml medium/flask).
† 50 mg of each acid listed.
‡ Added at expense of isonitrogenous amount of NH_4Cl.

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produced essentially same response as 50 mg of citric acid. Various additions had no effect on pH of medium nor did they increase growth over control when DNB was omitted.

Ketoacid content of mycelia grown under various conditions was determined in tissue rapidly frozen at –70°C. Ketoacids were converted to 2,4-dinitrophenylhydrazones, extracted and separated by means of their acid functions, and then isolated by paper chromatography(3). Identification was made by comparison of mobilities in at least 2 solvent systems in addition to characteristic colors produced by NaOH location reagent. Appreciable quantities of pyruvate and α-ketoglutarate were found to be normal constituents of young (up to 4 day) mycelia, but at 5 days no pyruvate was demonstrable, thus suggesting rapid metabolism of pyruvate by mature mold. However, when DNB (2.5×10^{-5} M) was added during first 48 to 72 hours of growth, resulting 5 day mycelia showed marked accumulation of pyruvate and little or no α-ketoglutarate. Thus it appeared that in the early growth phase pyruvate piled up because the oxidative pathway and cell synthesis were not maximal. Consequently, it seemed likely that the action of DNB was either (a) to prevent further metabolism of pyruvate, or (b) by preventing cellular proliferation, to produce an accumulation of pyruvate similar to that appearing in younger (slower growing) cultures. It is a well known characteristic of fungi to accumulate various carbohydrate breakdown products in presence of a relatively high concentration of substrate at a time when cell synthesis cannot, or is not permitted to, proceed at maximal rate(4).

To evaluate the interrelationship of certain carbohydrate metabolites and DNB, the dominant pathway of glucose utilization in the mold under these conditions was investigated. It was possible to obtain mycelial preparations with very low endogenous respiratory activity by using cultures incubated at 26°C for 53 hours on wrist-action shaking machine. Medium was the same as that already described except the glucose content was reduced to 0.5% and medium was 0.2 M with respect to phosphate buffer, pH 7. Mycelial pellets were harvested with suction, washed with water, pressed between blotting paper, and frozen for several hours at –20°C. (Both frozen mycelia and homogenates prepared therefrom were stored at –20°C for several days without loss of activity). Frozen material was ground with 3 volumes cold 0.1 M phosphate buffer, pH 6.0 in a Duall glass homogenizer and 0.5 ml added to Warburg vessels which contained 20 mg glucose in side arm and 0.2 ml 10% KOH on filter paper in center well. Total volume was made to 3.0 ml with buffer. After 10 min equilibration in atmosphere of air in 37°C bath, manometers were closed and, after a second 10 min period, glucose was tipped in from side arm and readings made at 10 min intervals for 1 hour. Oxygen uptake was linear for more than 1 hour under these conditions and in 60 assays averaged 1.55 μl per mg mycelium per hour. Oxygen uptake by identical preparations without glucose was less than 0.04 μl.

Glucose oxidation was unaffected by 0.01 M iodoacetate, iodoacetamide, fluoride, arsenite, malonate, cyanide or azide. The following substances were not oxidized: pyruvate, acetate, citrate, aconitate, α-ketoglutarate, glutamate, succinate and oxalacetate. Also, citrate and succinate did not alter rate of glucose oxidation. Fructose, ribose, arabinose and xylose were not oxidized. There was essentially no effect on observed oxygen consumption when the KOH trap for CO_2 was omitted from flasks (Table II). Dialysis at 1°C in pH 6 phosphate for as long as 22 hours did not affect activity nor did subsequent additions of DPN, TPN, thiamine pyrophosphate, ATP and cytochrome c. The molar ratio of glucose metabolized(5) to oxygen utilized was consistently close to 2. Lactic acid(6) was not formed. These same observations obtained with growth medium in place of phosphate buffer in Warburg flasks. Gluconic acid was identified as the main metabolite of glucose oxidation by subsequent chromatographic analysis of reaction mixtures (7). The principal acidic component of the mixture migrated with authentic gluconic acid in two different solvent systems and also reduced ammoniacal silver.

It seems clear, therefore, that metabolism of glucose proceeded overwhelmingly by

TABLE II. Glucose Oxidation under Various Conditions by *A. niger* Preparations. (Means ± stand. error.)

System	No. of exp.	Oxygen used	Glucose metabolized	Glucose / Oxygen
		μmoles/hr		
Control	16	9.51 ± .35	17.20 ± 1.57	1.84 ± .17
" minus KOH*	8	8.38 ± .53	20.50 ± 1.92	2.46 ± .18
Dialyzed control†	6	9.24 ± .09	23.87 ± 2.71	2.60 ± .31
Idem + cofactors‡	10	9.54 ± .11	23.38 ± 1.87	2.46 ± .21
DNB-treated cultures§	8	8.77 ± .71	18.47 ± 2.92	2.12 ± .22
Idem minus KOH*	8	8.53 ± .68	19.37 ± 2.97	2.31 ± .30

* KOH in center well replaced by H_2O.

† Preparations dialyzed 3 to 22 hr *vs* 0.05 M phosphate, pH 6.0 at 1°C.

‡ DPN, TPN, thiamine pyrophosphate, ATP, cytochrome c: 10 mg each/flask.

§ Cultures brought to 5.0×10^{-5} M DNB conc. 7 hr before harvesting.

either an Entner-Doudoroff pathway(8) or by direct conversion to gluconolactone and gluconate. Cleland and Johnson(9) have shown by use of labeled substrates that another strain of *A. niger* under similar conditions also converted glucose rapidly to gluconate and this was in turn slowly metabolized *via* pentoses to oxalate. Of further interest are data of Behal(10) which support the view that growth of *A. niger* is not dependent on the citric acid cycle and that this cycle constitutes only a minor respiratory mechanism in this mold.

Oxygen utilization and glucose/oxygen ratios of these preparations were not affected by DNB *in vitro* nor by addition of a growth depressing level of DNB (5 x 10^{-5} M) *in vivo* 7 hours before harvesting mycelial pellets (Table II). Activity of purified glucose oxidase (Sigma) was not influenced by DNB.

Discussion. An enzymic site for locus of DNB action is still obscure. It has been established that compounds such as isophthalate (*meta*), *m*-nitrobenzoate and *m*-halogenobenzoates function as competitive inhibitors of glutamic dehydrogenase due to their possession of required stereochemical and polar properties for inhibitor-enzyme bonding (11). Suggestion of a similar function for DNB would indeed be attractive because of critical importance of this enzyme in linking amino acid and carbohydrate metabolism. However, DNB had no influence on either forward or reverse reactions catalyzed by highly purified beef liver glutamic dehydrogenase.

Although dinitrophenol was about as effective as DNB in inhibiting growth of *A. niger* in the acid culture medium(1), inhibition due to the former compound was not affected by levels of carbohydrate intermediates which reversed DNB inhibition. Neither was dinitrophenol inhibition alleviated by concentrations of casein hydrolyzate or amino acids which completely overcame DNB growth inhibition. Results with media of different hydrogen ion concentration suggested that mycelial wall was impermeable to dissociated form of 2,4-dinitrophenol (pK = 4.0) since the phenol produced no inhibition near neutral pH. DNB was equally effective regardless of pH.

In view of apparently limited activity of citric acid cycle during growth in *A. niger* it is well to remember that inhibitory action of DNB is restricted to molds and its relative potency increases with decreasing age of cultures(1). These fungi assimilate remarkable quantities of glucose during growth and lay it down as structural polysaccharide. Therefore, it is thought that the action of such compounds as citrate, α-ketoglutarate, succinate and oxalacetate might be to supply metabolic intermediates possibly produced at an inadequate rate from glucose in presence of DNB. Furthermore, it seems a reasonable assumption that rate of formation of carbon rests *during growth* is critical even in absence of DNB because of near absence of citric acid cycle activity. Although these points require verification, evidence is taken to indicate that growth inhibition produced by DNB is mediated by way of depression of a normally marginal rate of biosynthetic formation of intermediates for amino acid synthesis.

Summary. Data suggest that major pathway of glucose oxidation in growing cultures of *A. niger* was *via* gluconate. The citric acid cycle played a minor role in young mycelium and inhibitory action of DNB appeared to involve further depression of formation of citric acid cycle intermediates with consequent decrease in rate of amino acid biosynthesis.

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Effects of Androsterone on Atherogenesis and Plasma Lipids in Cockerels. (26185)

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Testosterone propionate has been reported to inhibit coronary atherogenesis with simultaneous reductions in plasma cholesterol, phospholipid and cholesterol/phospholipid ratios in cholesterol-fed pullets(1). In the male bird Katz *et al.*(2) observed no effects on serum lipids and atherogenesis with small doses of testosterone. Higher doses significantly inhibited hypercholesterolemia, although aortic and coronary atherogenesis were not influenced(3). The effect on blood lipids, but not on atherogenesis, was in agreement with previous workers(4,5). The latter investigators reported decreased atherogenesis in testosterone-treated cockerels. The recent report by Hellman *et al.*(6) on hypocholesterolemic effects of androsterone in hyper- and normo-cholesterolemic patients stimulated further studies with androgens in experimentally-induced atherosclerosis. The present report compares effects of androsterone and testosterone propionate on atheroma and blood lipid shifts induced by cholesterol feeding in cockerels.

Methods. Three groups of Hy-line cockerels, 6½ weeks of age, were used for the study. Group 1 (control) was fed an atherogenic diet consisting of mash containing 2% cholesterol and 5% cottonseed oil. Daily subcutaneous injections of corn oil were given in volumes equivalent to those given drug treatment. Group 2 was fed similarly and injected daily with 3 mg/kg of androsterone dissolved in corn oil; Group 3 received the diet and 3 mg/kg of testosterone propionate in the oil solvent. Dosage of both compounds was adjusted each week to the changing weight of the birds. Plasma cholesterol and phospholipid concentrations, comb indices and food consumption were determined at 2-week intervals during the experimental regimen. Total cholesterol was determined by the method of Zlatkis *et al.*(7). The lipid phosphorus, determined by Sperry's method (8), was multiplied by the factor 25 to give the amount of total phospholipids. All birds were sacrificed and autopsied at the end of 8 weeks. The aortas were cut open longitudinally, fixed in 10% Formalin and stained with Sudan IV. Degree of aortic atherosclerosis was graded visually into 5 groups(0-4), according to the area of intima involved by le-

TABLE I. Effects of Androsterone and Testosterone Propionate on Plasma Lipids and Atheroma in Cholesterol-Fed Cockerels in an 8-Week Study.

Group	Plasma lipids: Cholesterol, mg %	Plasma lipids: Phospholipid, mg %	Atheroma: Aorta (0-4)	Atheroma: Coronary,† %
Control	1573 ± 134	594 ± 34	1.6 ± .2	45.1 ± 2.6
Androsterone	1353 ± 91	466 ± 26*	1.5 ± .2	25.9 ± 3.0*
Testosterone propionate	1376 ± 160	477 ± 44*	1.7 ± .1	30.6 ± 2.6*

* $P < .05$ that treat = control; values represent mean response (± stand. error) of 10 chicks /group.

† Proportion of arteries (in %) showing intimal lipid deposition; observations based on 23 to 84 arteries/heart.

sions. The hearts were fixed in Formalin and 4 equally spaced blocks were cut from each heart between the coronary groove and the apex. One frozen section was then cut from each block and stained with Sudan IV and hemotoxylin for histological examination of the coronary arteries. The index of coronary atherogenesis was based on the proportion of arteries showing presence of lipid in the intima. All evaluation for lesions in the aorta and coronary arteries was done under blind experimental conditions. The statistical significance of differences between mean values was determined by Student's t test.

Results. The group mean values for plasma lipids were not significantly different at 2, 4 or 6 weeks, hence only values for 8 weeks along with data on atherosclerotic lesions are shown in Table I. Plasma cholesterol levels for the androsterone and testosterone-treated groups were slightly lower but not significantly different from that of the control group. Both compounds, however, appeared to be approximately equipotent in reducing plasma phospholipid concentrations ($P<0.05$). By a primary effect on phospholipids, both steroids elevated the cholesterol/phospholipid (C/P) ratio, a metameter frequently used for description of relative shifts of the lipids in plasma. C/P ratios (mean ± S.E.) for control, androsterone and testosterone propionate were 2.60 ± 0.11, 2.90 ± 0.07 and 2.84 ± 0.19, respectively.

The severity of aortic atherosclerosis as judged by gross observation of the stained intima was similar for the 3 groups (Table I). The hearts appeared grossly normal. On microscopic examination, however, the hearts from both treatments exhibited significantly fewer arteries showing intimal lipid deposition.

Mean testicular weight of the androsterone-treated group did not differ significantly from the control group (Table II). As a contrast, treatment with testosterone induced a marked reduction in weight of the testes. Comb growth was stimulated to the same extent by androsterone and testosterone. No effect on body weight was observed with androsterone relative to the controls. Dosage of testosterone propionate used in this study, however, significantly depressed growth rate. Body weights (mean ± S.E.) for control, androsterone and testosterone propionate were 1.66 ± 0.03, 1.60 ± 0.05 and 1.39 ± 0.02, respectively. Measurements of food consumption revealed only negligible differences in intake.

Discussion. The most significant finding in the present study is that androsterone produced effects similar to those of testosterone propionate on plasma lipids and atherogenesis but without causing a reduction in testicular weight. The inhibition of cholesterol-induced *coronary atheroma* in the cockerel is apparently not related to lower plasma cholesterol levels since the treatments had no

TABLE II. Effects of Androsterone and Testosterone Propionate on Testicular Weight and Comb Growth in Cholesterol-Fed Cockerels.

Group	Testes, g	Comb growth,† mm
Control	9.1 ± 1.09	+ 89 ± 5.9
Androsterone	8.6 ± .83	+116 ± 3.7*
Testosterone propionate	.4 ± .04*	+117 ± 2.3*

* $P <.05$ that treat = control; values = mean response (± stand. error) of 10 chicks/group.

† Determined by change in length + height.

significant effect on this measurement. Such a relationship has been reported in pullets where testosterone propionate produced inhibition of coronary atheroma associated with a marked hypocholesterolemic response(1). The inhibitory effect of estrogenic agents has been reported to be associated with a decrease in C/P ratio; this reduction being due to a marked phospholipidemia with relatively little effect on cholesterol(9). It is therefore of particular interest that the inhibitory effects of the androgens used in this study were associated with decreases in plasma phospholipid and increases in C/P ratio. Recently Campbell *et al.*(10) reported that the weak androgenic agent, 19-norandrostenolone phenylpropionate, could produce significant regression of coronary atheroma without affecting plasma cholesterol or phospholipid levels in cholesterol-fed cockerels. These investigators postulated either a direct effect on the vessel wall or the anabolic (protein-conserving) activity of the compound may be playing a role in the protective action against experimentally-induced atheroma in the chick since increased dietary protein has been reported to have such a protective action(11, 12). Whatever the explanation may be, the present data support the concept that protection against coronary atheroma can be obtained with variable cholesterol and phospholipid changes in cholesterol-fed cockerels.

Summary. The effects of androsterone and testosterone propionate on atheroma and plasma lipid shifts induced by cholesterol feeding in cockerels were compared. Both compounds produced significant inhibition of coronary arterial atheroma. Aortic atherogenesis was not affected. The effects on plasma lipids were similar; non-significant reduction in cholesterol (C), significant decrease in phospholipid (P) with elevated C/P ratios. Testosterone propionate, but not androsterone, caused a significant reduction in testicular weight; both compounds stimulated comb growth. Further experimentation with androgens should provide useful information on the relationship between plasma lipid changes and inhibition of coronary atherogenesis.

The technical assistance of Astrida Kalnmalis and Margot Turner is gratefully acknowledged.

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Influence of Coagulases and Route of Injection on Staphylococcal Virulence in Mice.* (26186)

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The rare strains of staphylococci which lack either free coagulase or bound coagulase (the clumping factor responsible for agglutination and compact colony formation in fibrinogen), but not both these properties, have been considered to be of doubtful pathogenicity(1). However, Johanovsky(2) suggested that the clumping property rather than free coagulase may allow survival of staphylococci within phagocytes. Hunt and Moses (3) reported that only one of 2 serologically distinct variants of the Smith strain of *Staphylococcus aureus* was virulent for mice although both were coagulase positive. The mouse virulent variant formed diffuse colonies in plasma soft agar, whereas the colonies of the avirulent variant were compact in this medium. Alami and Kelly(4) concluded that the compacting of staphylococcal colonies in fibrinogen-containing soft agar is an expression of the clumping factor and need not be due exclusively to antibody action. The present study was initiated to compare free coagulase and bound coagulase as virulence factors and to determine whether route of injection affected the results in mice.

Materials and methods. Twelve strains representing typical coagulase positive and coagulase negative staphylococci as well as variants which lack either free or bound coagulase (hereafter referred to as coagulase and clumping factor, respectively) or both these properties were selected for study. The compact colony (S) and diffuse colony (Sv) forming variants of the Smith strain were kindly supplied by Dr. G. A. Hunt. Others were isolated locally from hospital personnel and out-patients or were variants of these strains. Coagulase and clumping factor activities were determined by the soft agar technic(4) as well as by conventional methods. Sterile filtrates of 18 hour 10% serum brain heart infusion cultures were used in coagulase titrations(5). Strains were also examined for other properties usually associated with pathogenicity, particularly hemolysins and staphylokinase. Four coagulase positive, clumping factor positive strains (S, V, E33 and H) comprised Group I. The 3 coagulase positive, clumping factor negative strains of Group II included the Sv strain and 2 previously unreported strains, K6 and K93, which have remained stable as to these properties since 1956. Strain K6 was isolated from the nasopharynx of a healthy carrier and K93 from a cutaneous ulcer. Three coagulase negative, clumping factor positive strains, Hvl, E35 and E35s, made up Group III. Hvl first appeared as a variant in soft agar cultures of the H strain. The other 2 are rough (E35) and smooth (E35s) forms of the same strain. Also tested as Group IV were 2 coagulase negative, clumping factor negative staphylococci, Hv2 and E33v, which are variants of H and E33, respectively.

Intravenous and intraperitoneal median lethal doses (LD_{50}) were determined by injecting an equal number of male and female, 20 to 30 g albino mice of CFW Webster Swiss origin with washed cells from 24 hour trypticase soy broth cultures. Cells were twice washed by centrifuging (2400 g) and resuspending the sediment in phosphate buffered saline. For each strain, 0.3 ml of each of 5 dilutions of a buffered saline suspension was injected intravenously into each of 6 mice and intraperitoneally into another group of 6 mice. Similarly, intraperitoneal challenge was repeated with cells suspended in 5% hog gastric mucin. Suitable controls included intravenous and intraperitoneal injection of 0.3 ml amounts of a sterile broth culture supernatant and a heat killed cell suspension of the highest challenge dose of each strain. The mice were observed for 14 days. At autopsy organs and body fluids

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TABLE I. Intravenous and Intraperitoneal Median Lethal Doses (LD_{50}) of Typical Staphylococci and Strains Which Lack Either Coagulase or Clumping Factor Activities.

Group	Strain	Coagulase titer	Clumping factor	LD_{50} Intrav.	LD_{50} Intraper. Without mucin	LD_{50} Intraper. With mucin
I	S	1:1024	+	4.5×10^8	1.1×10^9	1.2×10^8
	V	1:256	+	2.7×10^8	3.2×10^8	1.0×10^8
	E33	1:128	+	2.5×10^9	4.2×10^9	1.7×10^9
	H	1:64	+	6.4×10^8	2.8×10^9	1.6×10^8
II	Sv	1:256	—	4.0×10^7	1.1×10^7	1.0×10^4
	K6	1:8	—	1.8×10^9	2.1×10^8	6.3×10^5
	K93	1:8	—	1.7×10^9	1.8×10^8	1.1×10^5
III	Hv1	0	+	9.7×10^8	3.6×10^9	1.0×10^9
	E35	0	+	1.5×10^9	1.6×10^9	3.8×10^8
	E35s	0	+	8.5×10^8	2.8×10^9	3.9×10^8
IV	Hv2	0	—	9.4×10^9	3.2×10^9	3.2×10^8
	E33v	0	—	1.3×10^{10}	4.5×10^9	1.8×10^8

were examined microscopically and culturally.

Results. When injected intraperitoneally in 5% mucin not only the diffuse colony variant of the Smith strain (Sv), but also the other 2 coagulase positive, clumping factor negative strains of Group II (K6 and K93), were more virulent than organisms of other groups regardless of coagulase titer (Table I). There was no evidence that other properties of these strains explained their unusual pathogenicity.

The Sv strain proved to be the most virulent of all strains tested whether introduced intravenously or intraperitoneally, with or without mucin. The other 2 strains of Group II were no more virulent when injected intravenously than those of other groups, nor were their mucin-free intraperitoneal LD_{50} values significantly lower when all strains were compared by this challenge. However, small, though consistent differences were observed between the intravenous and mucin-free intraperitoneal LD_{50} of certain strains, notably those of Group II (Fig. 1). An overall analysis of variance of strain, group, route of injection, replicate and route of injection by group interaction indicates that only the last comparison is of statistical significance with $P < 0.01$. Although LD_{50} could not be correlated regularly with coagulase titer (*e.g.*, strains S and Sv), the coagulase negative, clumping factor negative variants Hv2 and E33v were slightly less virulent intravenously, but not intraperitoneally, than their coagulase positive counterparts H and E33. None of the control mice died.

Post-mortem cultures indicated that the character of the staphylococci injected remained stable in the infected mice. However, during the study, a mucoid variant (K93m) with a demonstrable extracellular slime layer was recovered occasionally, together with the non-mucoid parent organism, from mice injected with K93. The intraperitoneal LD_{50}

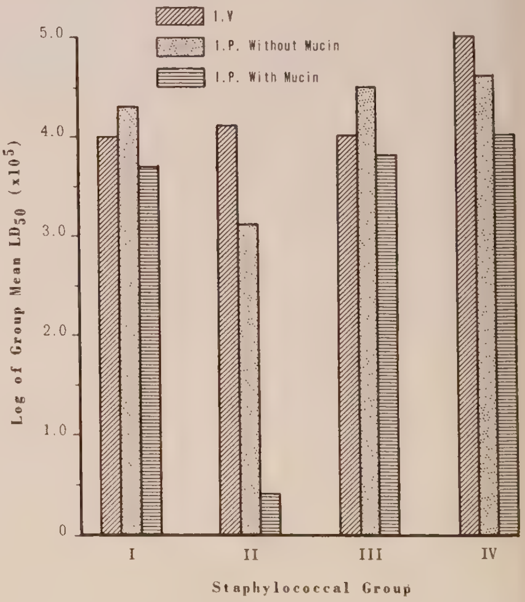

FIG. 1. Influence of route of inj. and mucin on mouse virulence of 12 strains of staphylococci grouped according to coagulase (C) and clumping factor (CF). Group I, C+, CF+; Group II, C+, CF–; Group III, C–, CF+; Group IV, C–, CF–.

of K93m was greater (6.2×10^8 without mucin; 1.2×10^7 with mucin) than that of K93 (1.8×10^8 without mucin; 1.1×10^5 with mucin), whereas intravenous LD_{50} values of both organisms were almost identical.

Discussion. The mouse is relatively resistant to infection with staphylococci and large numbers of these organisms are required to kill the animal. From the above results it is apparent that the intravenous and intraperitoneal median lethal dose was not significantly influenced by free coagulase, clumping factor or, indeed, by any other recognized property of the staphylococci. Kapral and Li (6) recently reported that *S. aureus* mutants which lacked either bound or soluble coagulase were just as virulent for rabbits when injected by the intradermal or intravenous route as was the parent strain which possessed both bound and free coagulase.

Reports of the influence of route of injection on susceptibility of mice to staphylococcal infection have been controversial(7). Unfortunately, this study does not provide conclusive evidence on this point. The fact that small differences between intravenous and mucin-free intraperitoneal LD_{50} were observed consistently in the case of certain strains, particularly those of Group II, suggests that similar studies should be extended to a larger series of staphylococcal strains and possibly to other genetic lines of mice. The clumping factor negative variant of the Smith strain (Sv) was the most virulent of all strains tested by either route of injection despite a lower coagulase titer than that of the clumping factor positive variant (S) of the same strain. Undoubtedly the "mouse virulent" Smith variant possesses some factor or factors yet to be identified.

The enhancing effect of mucin on virulence of coagulase positive, clumping factor negative staphylococci is in sharp contrast to that of the other strains tested. This finding agrees with that of Hunt and Moses with respect to the diffuse and compact colony forming variants of the Smith strain and it appears to be true of other coagulase positive staphylococci which lack the ability to agglutinate in presence of fibrinogen. These observations, together with the fact that mucin was more effective in decreasing LD_{50} of K93 than that of its mucoid variant, suggest the existence of a property or cellular component other than bound coagulase or extracellular slime which interacts with mucin in intraperitoneal challenge to affect virulence of staphylococci in mice.

Summary. Intraperitoneal and intravenous LD_{50} values of 12 strains of staphylococci, grouped according to free coagulase and the so-called clumping factor or bound coagulase, indicate that mouse virulence of *S. aureus* is influenced less by route of injection than by nature of the organisms injected. Mucin markedly enhanced mouse virulence for intraperitoneally injected coagulase positive, clumping factor negative staphylococci but not that of other strains. Neither free nor bound coagulase *per se* appeared to be a critical factor of virulence for mice.

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Sequence of Development of Hypercholesterolemia and Hypoproteinemia in Aminonucleoside Nephrosis. (26187)

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The nephrotic syndrome is characterized by proteinuria, hypoproteinemia and hypercholesterolemia. The mechanism involved in development of hypercholesterolemia is still unknown. Evidence relating the cholesterol changes to hypoalbuminemia has been presented by Rosenman, *et al.*(1) while others have challenged these concepts. Heyman and coworkers(2) showed that in experimental anti-kidney serum (AKS) nephrosis in rats, the cholesterol changes in the blood antedate the protein changes.

Since development of nephrosis following AKS is very rapid, (a matter of hours), it becomes difficult clearly to separate the time sequence of development of hypoproteinemia and hypercholesterolemia. It seemed worthwhole therefore to reevaluate this problem, using aminonucleoside nephrosis(3,4) as the experimental tool, since a latent period of at least 5 days exists prior to development of the disease, following which the lesion slowly evolves. It seemed likely that this prolonged time span might permit a sharper definition of the sequence of proteinuria, hypoproteinemia and hypercholesterolemia.

Experimental. Twenty-three male Sprague-Dawley rats with a starting weight of 100-120 g were studied. Animals were caged individually in metabolic units and provided food and water *ad lib.*

The nephrotic syndrome was produced in 15 animals with 11 daily injections of aminonucleoside(3,4) while 8 rats served as controls. Urinary protein excretion was measured daily from day 1-11 by the Esbach method(5). Blood samples were obtained daily, when possible, from tail veins of control and experimental animals. Total serum proteins were determined by the microtechnic of Lowry *et al.*(6) and cholesterol determinations were made by the micromethod of Saifer and Kammerer(7).

Results and discussion. Fig. 1 plots days of aminonucleoside injection and mean values of the 3 parameters measured for the entire group of nephrotic and control animals. It may be seen that proteinuria began by the fifth day, serum proteins began to decline by the sixth day and cholesterol levels rose definitively by the seventh day.

Sequential changes observed in individual injected animals were as follows: 1) The first change consisted in a simultaneous increase of urinary protein and decrease in serum protein in 9 rats; 2) proteinuria occurred first in 3 rats; 3) serum protein declined the day before proteinuria developed in 3 animals; and 4) 15 rats showed increased serum cholesterol levels as the third manifestation of the developing nephrosis.

These observations clearly show the almost simultaneous loss of urinary proteins and de-

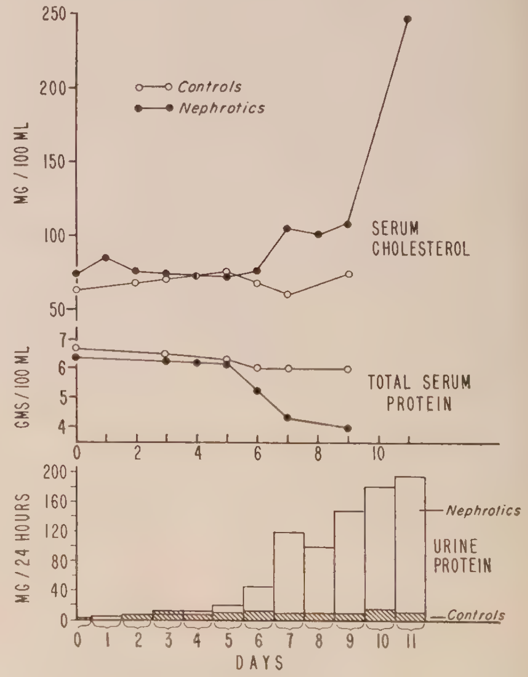

FIG. 1. Sequential changes in control and nephrotic rats during development of nephrosis.

crease in total serum protein as initial alterations in nephrosis. In all instances, a definite interval of from one to 4 days following proteinuria occurred prior to development of hypercholesterolemia. The sequence of hypoproteinemia and then hypercholesterolemia, though established here, does not necessarily indicate a causal relationship between the two.

Summary. The sequence of development of hypoproteinemia and hypercholesterolemia was studied in 15 aminonucleoside nephrotic animals. Hypercholesterolemia succeeds rather than precedes hypoproteinemia.

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The Juxtaglomerular Apparatus as an Extra-Adrenal Site of ACTH Action.*† (26188)

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We have shown that a variety of hypotensive drugs are able to produce morphological changes suggestive of hyperfunction in the renal juxtaglomerular apparatus(1). These responses occur within minutes to a few hours, in contrast to the weeks or months required for appearance of changes reported by others(2,3,4) and summarized by Tobian(5, 6). In attempting to explain the rapid responses to drugs which we have seen, we studied the relationship between the adenohypophysis and the juxtaglomerular apparatus (JGA). This report will indicate that JGA responds quickly to ACTH in absence of the adrenals and that it might be considered as an extra-adrenal target of this hormone.

Materials and methods. All experiments were performed on young male albino rats of the Harlan strain, weight approximately 150 g. Hypophysectomized rats were supplied by the Hormone Assay Laboratories. Adrenalectomized animals were maintained on 1% saline drinking water in addition to their regular rations. Some groups of rats were adrenalectomized 3 days after hypophysectomy, and were maintained on 1% saline drinking water plus their regular rations. In acute experiments, animals were used on the fourth post-operative day.

Drugs were administered intraperitoneally in doses indicated in the tables. The commercial ACTH used was Parke-Davis' brand of corticotropin. A specially purified ACTH which was virtually free of other endocrine activity was also used.‡ Other drugs used were from regular commercial sources. For hydralazine the solvent was 10% propylene glycol. Other drugs were dissolved in saline.

Radial wedges of kidney were removed at time of sacrifice, fixed in formol-sublimate-acetic acid fixative, and embedded in paraffin. Sections were cut at 6 μ and were stained by a combined Alcian blue-PAS technic. The method consisted in staining first

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† Presented in part at fall meeting of Am. Soc. for Pharmacol. and Exper. Therap., Ann. Arbor, 1958.

‡ Purified ACTH virtually free of other endocrine activity was supplied by research division of Parke-Davis Co.

with 0.15% aqueous Alcian blue for 30 minutes, followed by an alkaline alcohol wash for 1.5 hours. Next a conventional, periodic acid-Schiff stain was applied to the section. The method results in nuclei and cytoplasm stained faint green, brush border and basement membranes purple, and JG granules bright red. This provides better visualization of the granules than the PAS stain applied alone.

Degree of granulation was estimated in the juxtaglomerular apparati associated with 100 glomeruli from each animal. A weighted granulation index was calculated according to the method of the Hartrofts(7). Normal, solvent-injected, and sham-operated control groups were used. Each experimental and control group consisted of 6 animals. Experimental and appropriate control groups were compared statistically and reported as not significant if the P-value of the difference between means was 5% or more.

Results. The weighted granulation indices of 3 groups of control animals (Exp. 1, 2, 3) of Table I are not very different from one another, and agree closely with the control values reported by others(3). Removal of the adrenals produces significant elevation of JG granulation, as was reported by Dunihue(2), but this elevation does not occur in hypophysectomized animals (Exp. 4 and 5, Table I). Hypophysectomy alone produces no important change in the functional appearance of the JGA in a few days, but if the animals are maintained for 6 weeks a significant hypogranulation results, as shown in Exp. 6 and 7. The remaining experiments listed in Table I demonstrate that ACTH, both commercial and specially purified, produces marked hypergranulation of the JGA in intact rats, in adrenalectomized rats, and in hypophysectomized-adrenalectomized rats.

TABLE I. Effects of ACTH on Renal Juxtaglomerular Granulation in the Presence and Absence of Adrenals.

Exp. No.	Treatment	WGI*	P
1	Normal control	34 ± 3.5	
2	Sham-operated	39 ± 5.6	n.s.
3	Saline injected	37 ± 4.5	”
4	Adrenalectomized 4 days	51 ± 4.6	<5%
5	Hypophysectomized, then adrenalectomized 4 days	37 ± 1.3	n.s.
6	Hypophysect. 4 days	31 ± 5.2	”
7	” 6 wk	19 ± 4.2	<5%
8	ACTH 5 units i.p., 1.5 hr	95 ± 3.4	<1%
9	Adrenalect. 4 days then ACTH 5 units i.p., 1.5 hr	86 ±12	<2%
10	Adrenalect. 4 days, pure ACTH 5 units i.p., 1 hr	68 ± 4.2	<5%
11	Hypophysect.-adrenalect., then ACTH 5 units i.p., 1.5 hr	80 ±12	<1%

* Weighted granulation index ± stand. error.
n.s. = not significant.

TABLE II. Effects of Hypophysectomy on JGA Changes Produced by Drugs.

Exp. No.	Treatment	WGI*	P
1	Normal control	34 ± 3.5	
2	Hydralazine solvent control 0.5 cc i.p., 35 min.	39 ± 6.3	n.s.
3	Hypophysectomized, 4 days	31 ± 5.2	”
4	Hydralazine 25 mg/kg i.p., 35 min.	77 ± 3.2	<5%
5	Hypophysect. 4 days then hydralazine 25 mg/kg i.p., 35 min.	38 ± 5.2	n.s.
6	Vasopressin 1.25 units i.p. q 2 days × 3	77 ± 4.5	<5%
7	Hypophysect. 4 days then vasopressin 1.25 units i.p. q 2 days × 3	30 ± 3.2	n.s.

* Weighted granulation index ± stand. error.
n.s. = not significant.

Table II gives additional evidence for participation of the pituitary in the responses leading to morphological changes in renal JGA. The hypergranulation produced by such varied drugs(1) as hydralazine (Exp. 4) and vasopressin (Exp. 6) is seen to be completely prevented by prior hypophysectomy (Exp. 5 and 7).

Discussion. The action of ACTH in producing alterations of the JGA was first shown by Dougherty(8), who attributed this to an effect of the hormone on the adrenal cortex. It has also been shown that removal of the adrenals produces a hyperfunctional state of the JGA(2). Our results show that the effect of adrenalectomy upon the JGA cannot be a direct one, since it is prevented by prior removal of the pituitary. The effect is better explained by the increased circulating corticotropin level that follows adrenalectomy.

Garber *et al.*(1) have also shown that a variety of methods of raising ACTH level produce rapid alteration of the JG granulation.

The results of the experiments on the effects of ACTH, hydralazine, and vasopressin in the adrenalectomized and/or hypophysectomized animals indicate that ACTH has a direct tropic effect on the renal juxtaglomerular apparatus that is independent of the presence of the adrenals. The possibility that additional adenohypophyseal principles also affect the JGA has not been ruled out.

These observations may help to explain the diversity of procedures that have been shown to produce significant morphological changes in the JGA(2,3,4). These include alteration of salt balance, of adrenal function, of blood pressure. Finally, in connection with the recent suggested localization of renin to the JGA(9), Haynes *et al.*(10) found that ACTH stimulates renin release from the kidney, and that this action of ACTH also occurs in adrenalectomized animals. This is consistent with our suggestion that the JGA may be a target structure for the direct action of ACTH.

Summary. ACTH produces hypergranulation of the renal juxtaglomerular cells even in absence of pituitary and adrenal glands, and the chronic absence of ACTH depletes juxtaglomerular granules. The renal effect of vasopressin and of hydralazine, substances which produce hypergranulation of juxtaglomerular cells, is blocked by hypophysectomy. These findings support the concept that ACTH mediates many of the responses of the juxtaglomerular cells, and that the juxtaglomerular apparatus is an extra-adrenal site for the direct action of ACTH.

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An Improved *in vitro* Method for Determination of Serum "Insulin-Like" Activity. (26189)

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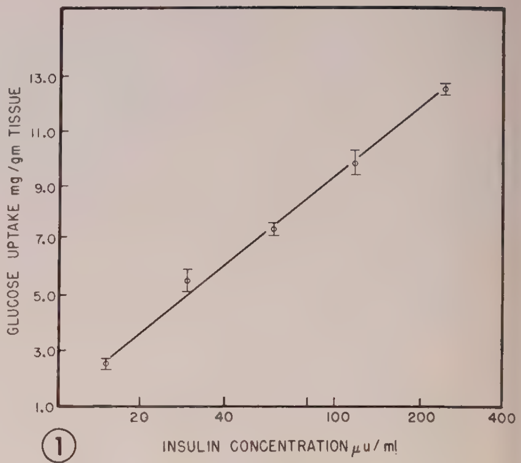
Accurate measurement of the insulin or "insulin-like" activity of biological fluids has been one of the major problems confronting endocrinological investigators. Much progress has been made since the introduction of the method utilizing uptake of glucose by the rat diaphragm. Vallance-Owen and Wright (1) have recently reviewed the available procedures. Martin *et al.*(2) have reported that the $C^{14}O_2$ production from glucose-1-C^{14} by the rat epididymal fat body was useful. Recently Beigelman(3) and Humbel(4) have utilized uptake of glucose by the epididymal fat body to measure insulin-like activity of human serum. In our studies we have modified the experimental design of the method using glucose uptake by epididymal tissue. This has resulted in a marked increase of accuracy and made the method readily amenable to statistical analysis. Although the present method was primarily designed to study the effects of various agents on the action of insulin, it should be extremely useful in determining insulin or "insulin-like" activity in biological fluids.

Methods. Male Holtzman rats (110-135 g) fed a standard laboratory diet were used. Food was withheld 2 hours prior to assay. Six animals were killed by decapitation and the epididymal fat bodies removed and placed in oxygenated Krebs-Ringer bicarbonate buffer(5) keeping the tissues from each animal in separate beakers. All handling of tissues was conducted with great care and as rapidly as possible. Eighteen 10 ml beakers were prepared, each with 2.0 ml of oxygenated Krebs-Ringer bicarbonate solution containing glucose (150 mg %), crystalline bovine serum albumin (250 mg%), and the appropriate amount of insulin or other substance to be assayed. In cases where sera were used, dilutions were made with oxygenated Krebs-Ringer Albumin Glucose (KRAG). It is highly desirable to determine concentration of glucose in the serum prior to bioassay and adjust the glucose concentration to approximately 150 mg%. It has been found feasible to reduce the incubation volume to 1.25 ml and thus conserve serum. Consequently, it is possible to obtain a 2-level assay on approximately 3 ml of serum using 1:3 and 1:12 dilutions. Eight pieces of tissue approximately 8-10 mg each were cut from the distal portion of each fat body and randomly distributed so that each of the 16 beakers had one piece of tissue from every animal. The design is similar to that used by Saffran and Schally(6) for *in vitro* assay of ACTH. Duplicate samples for each dosage level were used with 2 levels each of standard and unknowns with a 4-fold dosage increment. Two beakers contained only KRAG and served as controls for calculating uptake. With a little experience it was possible to obtain total tissue weights in each beaker within a range of ± 5-8 mg. The beakers, including the KRAG controls, were incubated in a Dubnoff metabolic shaker for 3 hours at 37-38°C using an oxygenation mixture of 95% oxygen and 5% carbon dioxide with a flow rate of 5 cubic feet per hour. At the end of incubation period the tissues were removed, blotted gently and weighed. Glucose concentrations of the incubation fluids were determined by the Somogyi-Nelson(7,8) procedure, or the ferricyanide method employing a Technicon Autoanalyzer. The latter has made it possible to double the number of assays with the same expenditure of time and personnel. Suitable control beakers should be run for each dilution of serum as well as the KRAG. Values were expressed as milligrams glucose uptake per gram of tissue.



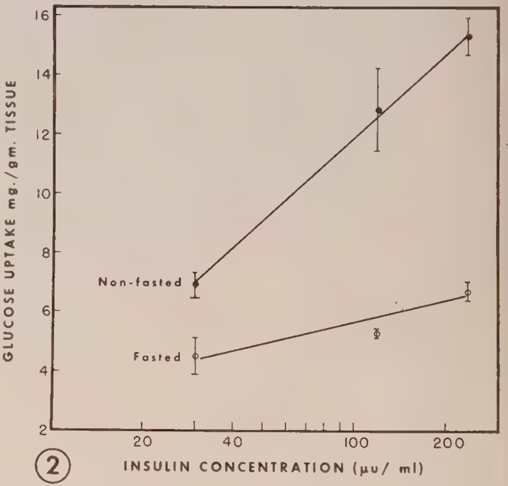


FIG. 1. Effect of insulin on uptake of glucose by the epididymal fat body. Individual points on curve represent avg response of duplicate samples together with indicated range.

FIG. 2. Effect of fasting on response of adipose tissue to insulin.

Results. Fig. 1 gives the dose response curve for an experiment using standard crystalline insulin (23.5 u/mg) at concentrations between 15 and 240 microunits/ml. It has been shown that higher concentrations give

TABLE I. Determination of Insulin-Like Activity in Dog Serum.

Animal No.	Serum dilutions	Insulin-like activity (μu/ml)	95% confidence limits	Index of precision (λ)*
1808	1:2, 1:8	178	105-251	.10
	1:2, 1:8	163	107-219	.08
1818	1:3, 1:12	148	118-178	.05
	1:3, 1:12	120	47-193	.14
1978	1:3, 1:12	120	50-190	.13
	1:3, 1:12	137	66-208	.12
	1:2, 1:8	149	41-257	.17

* $\lambda = \frac{s}{b}$.

essentially the same uptake as 240 microunits/ml. In fact, in many studies 120 microunits/ml was maximal. The tissue control (no added insulin) value in the above experiment was 2.26 mg/g. In other experiments we have found that sensitivity can be as low as 3.75 microunits/ml, particularly when uptake of glucose has been measured by means of uniformly labelled glucose-C^{14}. Experiments using glucose-C^{14} were found to parallel in all respects the results obtained with the above method.

To ascertain the usefulness of the assay in determining insulin-like activity in serum, 3 female beagle dogs were starved 16 hours and sufficient blood withdrawn from the jugular vein to conduct replicate assays on the sera. The sera were assayed for glucose and adjusted to approximately 150 mg %. Aliquots were then frozen for future assay. Table I summarizes the data on determination of the activity of the samples. Reproducible potencies with a high degree of precision can be obtained for a given sample. All of the assays cited were statistically valid in that there was no divergence in slope between the standard and unknown. In occasional samples a significant divergence can be noted and the assay has to be considered fundamentally invalid.

Assay limitations and calculations. On the basis of these data together with the previously reported validity of the response, it may be concluded that the method is useful for measurement of insulin or "insulin-like activity" with an excellent degree of precision.

To obtain pertinent information as easily as possible a simplified method of calculation has been devised, based upon the assumption that duplicate samples at each of 2 concentrations of standard and test preparation are employed and that the dosage increment is 4-fold. The factors used are constants valid only for these conditions. Factors for other conditions may be estimated from normal bioassay theory for symmetrical assays using the range to estimate standard deviation.

Let S_L, S_H, T_L, T_H = sum of duplicate responses of low and high doses of standard and test preparations respectively.

$$\text{Then } K = \frac{T_H + T_L - S_H - S_L}{T_H - T_L + S_H - S_L}$$

W = Sum of differences between duplicates of all doses.

$$I = \frac{\text{High Dose Std.}}{\text{High Dose Test}}$$

Relative Potency (R.P.) = I antilog 0.6 K

Index of Precision (λ)

$$\lambda = \frac{0.53\ W}{T_H - T_L + S_H - S_L}$$

$$V = \frac{W}{T_H - T_L + S_H - S_L}$$

$$F = 2.3\ V \sqrt{1 + K^2}$$

Confidence Limits (95%) = R. P. ± F (R. P.)

To test for parallelism between dose response curves of standard and unknown:

$$|P| = |T_H - T_L - S_H + S_L|$$

If $|P|$ is equal to or greater than $\frac{5W}{3}$, there is a significant lack of parallelism and the assay is fundamentally invalid. A significant lack of parallelism would indicate either that the response(s) for one of the preparations is outside the linear range of response, or that other factors such as inhibitors or potentiators are present which may modify the response of that preparation.

The usual index of precision (λ) for the assay is 0.15 or less. Occasional assays have given values of 0.05-0.07.

Discussion. To determine the validity of bioassays, it is important to be able to subject each assay to rigid statistical analysis. This is not possible with the majority of assays for "insulin-like" activity. In the case of the adipose tissue methods Beigelman(3) and Martin *et al.*(2) employ systems which compare response of one epididymal fat body to insulin and the other to the diluted or undiluted serum. This gives no information as to whether responses obtained in a given experiment are qualitatively similar. Serial dilutions of standard and unknown are necessary together with a measurement of the variability of the response. In the experimental design herein presented these factors have been taken into consideration and an assay method presented which is reproducible and accurate. A multiple dose level assay can be conducted on 3-4 ml of serum and a relative potency determined with confidence limits.

After this work was essentially completed Humbel(4) reported a method similar in design. Using animals fasted for 24 hours he found that the utilizable assay range under his conditions was 10-1000 microunits/ml in contrast to 15-240 microunits/ml reported here. From his data the assay slope was estimated to be 1.5-2.0. In contrast the authors analyzed 6 consecutive assays (non-fasted animals) conducted just prior to submission of this manuscript and found an average slope of 11.2 ± 1.2. A comparison of the responsiveness of epididymal fat bodies from 24 hour fasted and non-fasted animals was also conducted. Fig. 2 shows the marked difference in the slopes of the responses. The steeper slope increases the precision of the assay materially. The estimated lambda value $\left(\lambda = \frac{s}{b}\right)$ using Humbel's data was 0.3 or greater, whereas the present method consistently gave lambda values less than 0.15.

In addition to the length of fasting there were other differences: (a) the useful body weight range (110-135 *vs.* 120-160 g), (b) crystalline bovine serum albumin instead of gelatin in the incubation medium, (c) 150 mg % of glucose *vs.* 200 mg %, and (d) number of animals used for each assay (6 *vs.* 4). The use of 6 animals permits the potency determination of 3 samples per assay.

Summary. A highly reproducible and precise method for determination of "insulin-like" activity using glucose uptake by the epididymal fat body has been presented. It permits the assay of "insulin-like" activity in as little as 3 ml of serum with a calculation of relative potency with confidence limits as well as a test for assay validity.

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Properdin Titers in Sera from Germfree Rats.* (26190)

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Pillemer *et al.*(1) showed that normal human sera and other mammalian sera contain a protein, properdin, which in conjunction with complement and Mg^{++} constitutes a natural defense mechanism of the blood. They were of the opinion that properdin was a

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TABLE I. Properdin Titers in Sera from Germfree, Ex-germfree and Conventional Rats.

Germfree status		Ex-germfree status		Conventional rats		
		14 days	42 days			
Animal No.	Sample I	Sample II	Sample III	Animal No.	Sample II	Sample III
1	<1	2	8	10	2	4–8
2	<1	8	4	11	4	4–8
3	1	4	8	12	16	16–32
4	1–2	1–2	8	13	16	8–16
5	2	8	8	14	16	4–8
6	4	8	16	15	16	16
7	4	8	16	16	16–32	16
8	4	8–16	8–16	17	32	32
9	4	8–16	8	18	32	8

substance differing from antibodies. Pillemer(2) briefly mentioned that properdin was present in sera from germfree rats.

In this paper the properdin titers of germfree rats are compared with those of normal rats of the same strain. The properdin titers of the germfree animals were also studied after the animals had been transferred to the animal room and contaminated with the microbial flora from conventional rats.

Material and methods. The germfree rats were reared according to Gustafsson(3,4). Animals from 5 different litters of the 9th and 11th generations of the inbred germfree strain were used. Nine germfree animals of both sexes and aged 125 days were compared with 9 conventional rats aged 125-140 days. All animals had been reared on the autoclaved diet D_7(4) on raised screens. This diet is not antigen-free, since it contains casein and a number of dead bacteria. About 1.5 ml of blood was sampled from germfree animals when still in the germfree apparatus. They were afterwards transferred to the animal room and given an enema of 1 ml of a suspension of cecal contents from a conventional animal. After 14 days and 42 days new blood samples were collected from these previously germfree animals and from control animals.

The blood specimens were allowed to stand at room temperature for 2 hours and then centrifuged. The serum was afterwards drawn off and frozen at –60°C.

Properdin was determined according to the zymosan assay of Pillemer *et al.*(5) The reagents necessary (RP and R3) were prepared from human, pooled sera according to instructions given by Pillemer *et al.*(5). Before use these reagents were tested with 5 normal human sera which have been used as our standard sera for this purpose for the last 2 years. All samples were studied on the same day to exclude variations due to zymosan and sensitized sheep erythrocytes. The results were expressed in units described by Laurell(6).

All rat sera were diluted ¼. When properdin titers were found to be less than 4 units sera were also studied undiluted. No test was performed with the modified zymosan-assay(6) because the amounts of serum obtained from blood samples were not large enough to permit both tests.

Results. Table I shows that properdin titers of germfree animals were originally significantly lower than those of conventional animals, and that after exposure of the animals to a conventional environment (sample II and III) the titers rose to about the same levels as those of conventional animals.

Discussion. Using electrophoretic as well as immunologic technics Gustafsson & Laurell(7,8) found significantly lower concentrations of gamma- and beta-globulins in germfree rats than in control groups of normal rats, while the other serum fractions were within normal limits. Gamma-globulin values of sera from ex-germfree animals gradually rose but did not reach normal level until about 2 months after the animals had left the germfree environment. No changes were found in albumin and alpha-globulin levels 14 days after exposure of the germfree animals to a conventional environment. The germfree rats might therefore be regarded as healthy, suggesting that the normal flora of micro-organisms are an important stimulant of gamma-globulin production.

The germfree rats studied with respect to serum properdin content belonged to the same strain and were reared under the same conditions as those of the series of Gustafsson & Laurell(7,8). The germfree rats in the present investigation may therefore be regarded as hypo-gamma-globulinemic. The plasma homeostasis of properdin, like that of the gamma-globulins, seems to be regulated by the normal bacterial environment. In this respect, therefore, properdin falls in the same category as the gamma- and beta-globulins.

Pillemer *et al.*(1) were of the opinion that properdin was a substance not related to antibodies. On the other hand evidence is accumulating(9,10,11,12,13) that properdin may not be a single, unique substance, but represents a group of normal antibodies crossreacting with antigens present *i.a.* in zymosan.

Pillemer(2) showed that sera from germfree rats possessed properdin when tested in the zymosan assay. Pillemer's data have been published more in detail by Wagner (14). Properdin values of these germfree animals were on the same level as those of the conventional rats. This might *per se* suggest that properdin is not an antibody provided, however, that the animals were not stimulated by antigens. That this might be the case in the referred investigation is suggested by the fact that the animals showed a considerable titer of antibodies against *M. epidermidis*.

Osawa & Muschel(13) reported that the sera of 6-week-old germfree guinea pigs were comparable in bactericidal activity against *Sh. dysenteriae* and *S. typhosa* strain 0901 to that of the sera of conventional animals. They pointed out that these findings were not conclusive, since "the germfree animals are not free of antigenic stimulation nor of passively transmitted maternal antibody."

Neither could the possibility be excluded in our series that antigenic stimulation of the germfree animals by diet or by dead bacteria in the food occurs. However, based on the significantly decreased gamma- and beta-globulin levels in the sera of the presently studied strain of germfree rats it might be assumed that antigenic stimulation of the germfree animals—both with respect to intensity and to number of different antigens encountered—is much weaker than that of conventional animals. This might explain the low properdin values obtained in the zymosan assay with sera from germfree animals, as possibly only a few of the globulins or antibodies crossreacting with the antigens of zymosan may have been formed or stimulated to increase in titer. Under the influence of a normal bacterial flora the properdin of the ex-germfree animals gradually approach normal levels like the gamma-beta globulins.

Summary. 1. The properdin content of sera from germfree rats is lower than that of conventional animals. 2. Properdin titers of germfree animals gradually reach normal values after exposure to a conventional environment. 3. It is concluded that properdin level, like gamma-globulin level, is influenced by the normal bacterial flora.

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Action of Foot-and-Mouth Disease Virus and Metabolic Poisons on Bovine Kidney Culture Cells. (26191)

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Viral propagation is known to be dependent on host cell metabolism. Carbohydrate metabolism is important both as a source of energy and of carbohydrate intermediates which may serve as precursors in the viral synthetic process. Virus-induced increases in glycolysis have been observed with poliovirus (1), adenovirus(2), Newcastle disease virus (3), and foot-and-mouth disease virus (FMDV)(4), while infection with influenza virus has been reported to inhibit glycolysis of white blood cells(5). The importance of metabolic activities in the citric acid cycle has been demonstrated for influenza virus by deprivation of substrates, suppression of oxygen tension, interruption of the hydrogen transport system, and inhibition of a primary dehydrogenase(6). Development of poliovirus in mice has been inhibited by the presence of fluoroacetate which functions as a blocking agent of the citric acid cycle(7), but was not inhibited in HeLa cells by several amino acid and purine analogues(8). Anaerobic maintenance of host cells has inhibited the replication of mouse encephalomyelitis virus(9), and pneumonitis virus(10), while poliovirus was able to replicate(11).

The study reported here concerns the effect of various metabolic poisons on normal and infected bovine kidney culture cells. The poisons used are inhibitors of glycolysis, the citric acid cycle, and the cytochrome oxidative sequences. Infected cells are maintained aerobically and also anaerobically.

Materials and methods. Tissue culture. Primary monolayer cultures from bovine kidney tissue were prepared in 4-oz. prescription bottles as previously described(12). The growth medium consisted of 0.5% lactalbumin hydrolysate and 2% bovine serum in Hanks' balanced-salt solution. *Virus.* High-titer stocks of FMDV-A119 were produced from infected tissue culture fluids by precipitation with methanol, and the alcohol removed by subsequent dialysis(4). *Virus assay.* Virus assays were done by the plaque method using the monolayer cultures in prescription bottles(12). *Respiration.* Cell suspensions were prepared from cells scraped from the prescription bottles by packing them at 300 g for 5 minutes and resuspending in growth medium at concentrations of about 25×10^6 cells per ml. Warburg flasks contained 1 ml of cell suspension, 1 ml of isotonic Tris buffer, pH 7.5, containing the poison to be tested, and when needed, 0.50 ml of viral concentrate at 5-10 plaque forming units (PFU) per cell. Oxygen uptake was measured at 37°C with 0.20 ml of 10% KOH in the central well. *Virus production.* Cell layers in prescription bottles were rinsed twice with fresh portions of Tris buffer, then inoculated with 0.125 ml of viral concentrate (4-6 PFU per cell) and 0.125 ml of Tris buffer containing twice the desired final concentration of poison to be tested. At 90 minutes, the cells were washed 5 times with 5 ml portions of Tris buffer to remove most of the unadsorbed inoculum, and reincubated with 5 ml of growth medium-Tris buffer (1:1) containing the poison. At the desired time interval (100-150 minutes post inoculation for initial appearance of virus, and at 300 minutes for total virus production), 5 ml of 1% sodium dodecyl sulfate in Tris buffer was added to lyse the cells. The resulting lysate, which contained intracellular as well as extracellular FMDV progeny(4,13), was assayed for virus. In those experiments requiring an anaerobic environment, the prescription bottles were evacuated and flushed 5 times with nitrogen, then kept tightly stoppered. *Glucose-lactic acid.* Cell layers were inoculated as for virus production, but instead of the rinse at 90 minutes, 2.25 ml of growth medium-Tris buffer (1:1) containing the poison being tested were added and the bottles were rocked gently until 210 minutes post inocula-

TABLE I. Effect of Metabolic Poisons on Respiration of Suspensions of Kidney Culture Cells.*

Exposure time (hr)	% of control (no poison) respiration†							
	Iodoacetate		Fluoride		Malonate		Fluoroacetate	
	10^{-4}M	10^{-3}M	10^{-3}M	10^{-2}M	10^{-2}M	10^{-1}M	10^{-3}M	10^{-2}M
1	100	94	100	95	100	77	89	78
2	100	81	100	98	100	77	83	77
3	100	70	100	93	100	74	79	73
4	100	60	100	89	100	71	75	70
5	100	51	100	83	100	65	71	66

* Data are for both normal and infected cells.

† There was also no difference in respiration between normal and infected cells during first 5 hr after infection, in the absence of any poison. Normal respiration was 30 μl of oxygen/hr/ 25 × 10^6 cells(4).

tion. A 2.0 ml aliquot was then removed and treated with an equal volume of 10% tri-chloroacetic acid. The resulting supernatant liquids were assayed for glucose by the anthrone reagent(14) and for lactic acid(15). *Metabolic poisons.* The chemicals were used as the sodium salts. Six replicates were done for chemical and viral asays, and 4 for respiration measurements.

Results. The effects on respiration of normal and infected cells in suspension exposed to varying concentrations of 4 different metabolic poisons were identical (Table I). 10^{-4}M iodoacetate, 10^{-3}M fluoride, and 10^{-2}M malonate had no effect on respiration over a 5-hour exposure period. Ten-fold higher concentrations of the same agents decreased respiration rates in all cases, the effect being progressive with 10^{-3}M iodoacetate and 10^{-2}M fluoride, while 10^{-1}M malonate caused a rapid initial drop in respiration followed by a slow continued decrease. Fluoroacetate also caused a relatively large initial drop with comparatively small differences in effect between the two concentrations used.

The effect of cyanide and azide was measured by extent of cell loosening from the glass culture vessel instead of attempting to maintain the poisons in the Warburg flasks in the recommended way(16). 10^{-2}M concentrations of these poisons did not influence cellular attachment to glass over the 5-hour period studied. At 5-fold higher concentrations, azide still had no effect by 5 hours, while cyanide caused partial cell loosening in 3 hours and complete cell loosening within 4 hours.

Table II gives the effect of 2 known poisons of the glycolytic cycle, iodoacetate and fluoride, on glucose utilization and lactic acid production in both normal and infected cells. Iodoacetate, at 10^{-3}M concentration, stopped glycolysis immediately in both classes of cells, while 10^{-4}M iodoacetate decreased glycolysis by half in less than 30 minutes, and thereafter to less than 5-6% of control values. Fluor-

TABLE II. Effect of Iodoacetate and Fluoride on Glucose Utilization and Lactic Acid Production of Bovine Kidney Cultures Cells, Normal and Infected. Percentage of control (no poison) values.*

Exposure time (min.)	Iodoacetate				Fluoride			
	10^{-4}M		10^{-3}M		10^{-3}M		10^{-2}M	
	Glucose	Lactic acid	Glucose	Lactic acid	Glucose	Lactic acid	Glucose	Lactic acid
Normal cells:								
30	—	53	—	0	—	100	—	85
210	10.5	9.1†	0	0	97	100	65	69
Infected cells:								
30	—	58	—	0	—	100	—	87
210	8.4	8.0†	0	0	95	100	42	46

* Control values, μg/hr/million cells, for normal and infected cells, respectively, were: glucose, 9.6 and 11.8; lactic acid, 5.3 and 10.2.

† There is still lactic acid production after first 30 min., equal to about 5 to 6% of control values.

TABLE III. Effect of Metabolic Poisons on Initial Appearance of Virus and Its Total Production in Bovine Kidney Culture Cells.

Poison	Virus yield, PFU/ml (log units)	Delay in initial virus appearance (min.)	Poison	Virus yield, PFU/ml (log units)	Delay in initial virus appearance (min.)
Normal control	7.3	—	Normal control	7.3	—
Iodoacetate,			Malonate,		
10^{-6}M	7.3	—	10^{-2}M	7.3	none
10^{-5}M	7.0	none	2.5×10^{-2}M	7.3	—
10^{-4}M	6.3	"	5.0×10^{-2}M	7.0	—
10^{-3}M	4.3*	—	1×10^{-1}M	6.7	10
Normal control	7.3	—	Normal control	7.8	—
Fluoride,			Fluoroacetate,		
10^{-3}M	7.3	none	10^{-4}M	7.8	—
5×10^{-3}M	6.4	—	1×10^{-3}M	7.8	0–10
1×10^{-2}M	5.8	none	5×10^{-3}M	7.3	—
5×10^{-2}M	4.4*	—	1×10^{-2}M	6.8	30
Normal control	7.3	—	Normal control	7.3	—
Cyanide,			Azide,		
10^{-3}M	7.1	0–10	10^{-3}M	7.2	0–5
5×10^{-3}M	6.6	—	5×10^{-3}M	7.1	—
1×10^{-2}M	6.0	30	1×10^{-2}M	6.4	40
5×10^{-2}M	4.2*	—	5×10^{-2}M	4.1*	—

* No virus production; titer represents residual viral inoculum(4).

ide, at 10^{-2}M concentration, decreased glycolysis to about two-thirds of control values in normal cells and had a greater effect in the virus infected cells, while 10^{-3}M fluoride had no appreciable effect on glycolysis in both normal and infected cells.

Table III gives the effect of the metabolic poisons on virus production in cells. Iodoacetate, at 10^{-3}M concentration, stopped all virus production; 10^{-3}M fluoride had no effect; 10^{-2}M fluoride lowered virus production by about 1.5 log units. Neither poison influenced time of initial appearance of virus. Fluoroacetate showed a stronger inhibitory effect than malonate on a molar basis, and both poisons delayed the onset of virus production. Cyanide showed a slightly more inhibitory effect than azide, and both poisons also extended the latent period of virus production.

Table IV shows the effect on virus production when the poisons were present only at selected time intervals during the virus growth cycle. The concentrations of poisons which decreased virus production when present throughout had a lesser effect when present only during the time period of 90-300 minutes. The effect of the presence of the poison solely during the first 90 minutes was negligible except in the case of iodoacetate. Absence of oxygen did not interfere with virus production.

Discussion. The poisons used fall into 3 categories based on the metabolic sequences they inhibit. Iodoacetate and fluoride affect enzymes of the glycolytic cycle, inhibiting triose phosphate dehydrogenase and enolase, respectively. Fluoroacetate and malonate affect enzyme substrates of the citric acid cycle, interfering with oxidation of citrate and of succinate, respectively. Cyanide and azide react with the heme containing enzymes of the cytochrome oxidation sequences. The virus itself was found to be stable in presence of all the poisons tested.

Decrease in respiration rate and extent of cell loosening can be used as a measure of the compatibility of the cells with the poisons present. The cells showed remarkable resistance to all the poisons used; respiration of the cells may be influenced more by other metabolic cycles than the citric acid cycle. Iodoacetate had the strongest effect, which may be explained by the fact that the poison is a non-specific inactivator of sulfhydryl groups, the integrity of which are necessary

TABLE IV. Effect on Virus Production of Poisons Present in Host Cells for Different Times during Virus Growth Cycle. Effect of anaerobic environment on virus production.

Poison		Normal control	Virus titer, PFU/ml (log units)		
			Present 0-300 min.	Present only 0-90 min.	Present only 90-300 min.
Iodoacetate,	1×10^{-4}M	7.7	6.6	7.0	6.9
	1×10^{-3}M	7.7	4.3*	5.4	5.5
Fluoride,	1×10^{-3}M	7.3	7.3	7.3	7.3
	1×10^{-2}M	7.7	6.1	7.6	6.5
Malonate,	1×10^{-2}M	7.3	7.3	7.3	7.3
	5×10^{-2}M	7.7	7.5	7.7	7.7
	1×10^{-1}M	7.3	6.6	7.3	7.1
Fluoroacetate,	5×10^{-3}M	7.6	7.1	7.4	7.3
	1×10^{-2}M	7.6	6.7	7.4	7.3
Cyanide,	1×10^{-3}M	7.4	7.3	7.4	7.4
	1×10^{-2}M	7.5	6.3	7.5	7.2
Azide,	1×10^{-3}M	7.5	7.3	7.5	7.5
	1×10^{-2}M	7.5	6.7	7.5	7.2
Nitrogen		7.5	7.5	7.5	7.5

* No virus production; titer represents residual viral inoculum(4).

for the action of numerous enzymes. The action of iodoacetate was also tested in presence of 10^{-2}M pyruvate. No change was noted. The decrease in respiration was therefore not caused by lack of pyruvate coming from the glycolytic scheme to be used in the citric acid cycle. Malonate had the least effect upon respiration with no effect at all at 10^{-2}M concentration. The impaired respiration with 10^{-1}M malonate may be attributed to the general effect of high salt concentration and not to a specific action. It would seem the bovine kidney culture cells may follow other oxidative sequences in addition to the citric acid cycle. Virus infection had no influence on respiratory response of the cells to the poisons, indicating that the presence of the virus did not weaken further the already damaged cellular systems.

Iodoacetate had a stronger effect than fluoride on glycolysis, and showed no difference in its action in the presence of virus. Fluoride, however, caused a greater decrease in glycolysis in virus-infected cells than in normal cells, which may indicate that virus infection had either changed or weakened a metabolic sequence affected by fluoride. Addition of pyruvate did not affect the action of either poison.

Virus production was affected more strongly by the glycolytic poisons, particularly by iodoacetate, than by any of the other groups of poisons. Only iodoacetate exercised a strong effect when present only during the 0-90 minute period after infection of the cells with virus. This may indicate that the effect of iodoacetate is irreversible, or that iodoacetate blocks all stages of viral replication—adsorption, penetration, synthesis and asembly of subunits, and discharge of intact virus. The action of the other poisons is apparently more reversible. It is of interest that the glycolytic poisons showed no effect on the latent period of virus production, while cyanide and azide produced a definite increase in latent period which may be interpreted as interference with the energy producing mechanisms required for virus synthesis. This interference may be associated with any heavy metal containing enzymes and not necessarily with the cytochrome oxidation chains. The greater effect on virus production shown by fluoroacetate as compared to malonate makes it probable that some other factor than interference with the oxidative steps of the citric acid cycle is involved. It has been reported that fluoroacetate causes a fall in concentration of free amino acids in treated tissues(17), and the effect of fluoroacetate upon viral propagation probably results from the diverting of viral precursors into synthesis of citrate(6).

The lack of effect of absence of oxygen during the 5-hour period of virus production is a further indication that oxidative sequences are not as vital to production of virus as are glycolytic sequences of the host cell.

Summary. Bovine kidney culture cells were found to be resistant to high concentrations of poisons of the glycolytic cycle, the citric acid cycle, and the cytochrome oxidation chain, as measured by respiration and cell loosening from the glass. Infection with FMDV had no influence on the results, and it may be that respiration of the cells is more dependent on other metabolic cycles. Iodoacetate and fluoride, present in concentrations which caused large decreases in glycolysis, decreased virus production more than any other poisons. Malonate had an insignificant effect on virus production, while the larger effect of fluoroacetate was probably due to its action in removing viral precursors. The fact that maintenance of infected cells in an anaerobic environment did not affect virus production was a further indication that glycolytic sequences of the host cell are more essential to viral replication than oxidative sequences.

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Studies of *Mycobacterium tuberculosis* $H_{37}Rv$ Grown *in vivo:* Inhibitor of Lactic Acid Dehydrogenase in Normal and Infected Mice. (26192)

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It has been previously reported(1,2) that intact cells and cell-free extracts of tubercle bacilli grown *in vivo* ($LH_{37}Rv$) inhibited lactic dehydrogenase in cell-free extracts of *Mycobacterium tuberculosis* $H_{37}Rv$, *Mycobacterium tuberculosis* BCG and *Mycobacterium phlei* as well as succinoxidase of lung homogenates of normal mice. Similar inhibition was displayed by lung extracts from mice infected with $H_{37}Rv$, but not by those from normal mice. The question arose whether the inhibitor was being produced by the tubercle bacillus itself or by the host tissues. Since the problem of the origin of the inhibitor is of great importance, it seemed essential to ascertain whether lung extracts from normal mice are completely devoid of inhibitory activity. Our findings that lung extracts from normal mice did not display any inhibitory activity do not preclude the possibility that the inhibitor in question was present in them but in quantity so small as not to be detected under our previous experimental conditions. This paper is concerned with the elucidation of this problem.

Materials and methods. The organism used was *Mycobacterium phlei.* The cells were grown at 37° on a rotary type shaker in a

Difco Nutrient Broth supplemented with Tween-80, 0.5%; sodium chloride, 0.5%; and K_2HPO_4, 0.25%. Three-day-old cultures were used. The cells were maintained on slopes with solid medium of a similar composition except for Tween-80. The cells of *M. phlei* were collected by centrifugation, washed twice in a 0.1M phosphate buffer, pH 7.2, and disintegrated in a 9 KC Raytheon magnetostriction oscillator for 45 minutes. Cell debris and broken cells were removed by centrifugation at 7700 × g for 10 minutes in the cold. The supernatant was used throughout. Protein content of the cell-free extracts was estimated by the biuret method. Mice were infected with *M. tuberculosis* $H_{37}Rv$ by the method described previously(2). For preparation of extracts from infected and normal mice, 2.5% homogenates were used. The various organs were homogenized in distilled water in a teflon homogenizer run at top speed for 2 minutes. The homogenates were then spun at 10,000 rpm in an MSE high speed centrifuge and the reddish opalescent supernatant was used. Dehydrogenase activity of cell-free extracts of *M. phlei* was determined by reduction of triphenyltetrazolium chloride under aerobic conditions. Formazan was extracted with *iso*-butanol and its color intensity read in a Coleman Jr. spectrophotometer at 485 $m\mu$. Values are expressed in O.D. of formazan.

Results. Inhibition of lactic acid dehydrogenase of M. phlei *by lung extracts from normal mice.* To determine whether lung extracts from normal mice contain an inhibitor of lactic dehydrogenase of *M. phlei* we have performed a series of experiments using: 1) large amounts of lung extracts, 2) cell-free extracts of *M. phlei* of low dehydrogenase activity, and 3) preincubation of lung extracts with cell-free extracts of *M. phlei* before determining ~~their~~ lactic dehydrogenase activity.

1) Experiments with large amounts of lung extracts from normal and infected mice. In this series of experiments we determined the effect of increasing amounts of lung extracts from normal and infected mice on lactic dehydrogenase of cell-free extracts of *M. phlei*. The results (Table I) show that lung extracts from normal mice inhibit lactic dehydrogenase activity of cell-free extracts of *M. phlei* when used in amounts 2-3 times higher than those from infected animals. Six-tenths ml of lung extract from normal mice caused a 42% inhibition of lactic dehydrogenase of cell-free extracts of *M. phlei,* while a similar amount of lung extract from mice infected with $H_{37}Rv$ brought about an inhibition of 68%. Furthermore, it can be seen that the inhibitory activity of lung extract from infected mice is detectable at 2-3 fold higher dilutions than that of extracts from normal mice.

TABLE I. Effect of Various Amounts of Lung Extracts from Normal and Infected Mice on Lactic Dehydrogenase Activity of Cell-Free Extracts of *M. phlei.*

	Reduction of tetrazolium by cell-free extracts of *M. phlei* in presence of			
	Lung extracts from infected mice		Lung extracts from normal mice	
Amt of lung extracts used, ml	O.D. of formazan	Inhibition, %	O.D. of formazan	Inhibition, %
none	1.00		1.00	
.60	.32	68	.58	42
.40	.32	68	.74	28
.30	.42	58	1.00	0
.20	.66	34	1.10	stim*
.10	1.00	0	1.10	"

Reaction mixture contained: 6 mg protein of *M. phlei* extracts in 0.1M phosphate buffer pH 7.2, 0.5 ml; 0.3M sodium lactate, 0.1 ml; 1% aq. sol. of triphenyltetrazolium chloride, 0.1 ml; lung extracts as indicated. Final vol 2.2 ml. The reaction was stopped after 10 min. incubation at 37°.

* Stimulation.

2) Experiments with cell-free extracts of *M. phlei* of low dehydrogenase activity. It has been observed during these experiments, that lung extracts from normal mice inhibited lactic dehydrogenase in some batches of *M. phlei* preparations even when the former were used in small amounts (0.25 ml of lung extract per reaction mixture). The inhibition became apparent only with cell-free extracts of *M. phlei* of low dehydrogenase activity, (less than 0.7). Activity of lactic dehydrogenase is expressed in O.D. of formazan formed by cell-free extracts of *M. phlei* in presence of lactate in 10 minutes. In view of the above findings, experiments were designed to determine whether depending on the potency of *M. phlei* dehydrogenase, a

TABLE II. Effect of Lung Extracts from Normal and Infected Mice on Lactic Dehydrogenase Activity of Cell-Free Extracts of *M. phlei* of Various Enzymatic Activities.

Dehydrogenase activity of cell-free extracts of *M. phlei*		
Control	In presence of 0.25 ml of lung extract from normal mice	In presence of 0.25 ml of lung extract from infected mice
1.50	2.70	.90
1.00	1.15	.32
.50	.15	.07

Conditions as in Table I.

given amount of lung extract may either stimulate, inhibit, or have no effect on the action of the enzyme. The results showed this to be the case. In the experiment given in Table II we determined the effect of a given amount of lung extract from normal and infected mice on lactic dehydrogenase of cell-free extracts of *M. phlei* of various enzymatic activities. These were obtained by dilution of the enzyme. Activity of the undiluted enzyme was 1.5 and that of the 2 dilutions 1.0 and 0.5 respectively. A given amount of lung extract from normal mice stimulated the lactic dehydrogenase of high enzymatic activity/ 1.5/, had no effect when the enzymatic activity was 1.0, and exhibited a pronounced inhibitory effect on the same enzyme when activity of the latter was lowered to 0.5. On the other hand, lung extract from infected mice in amounts similar to those from normal animals inhibited even the most active preparations of lactic dehydrogenase of *M. phlei*.

3) Experiments on preincubation of cell-free extracts of *M. phlei* with lung extracts. The results described above show that there is an interplay between amount of inhibitor used and activity of lactic dehydrogenase. Presence of the inhibitor in lung extract from normal mice can be revealed either by using large amounts of the aforementioned extract or by using cell-free extracts of *M. phlei* of low dehydrogenase activity. The experiments to be described below show that it is possible to detect the inhibitor in lung extracts from normal mice using small amounts of lung extracts and cell-free extracts of *M. phlei* of high enzymatic activity. This can be achieved by preincubation of cell-free extracts of *M. phlei* with the lung extract before determining dehydrogenase activity of the former. In these series of experiments we incubated cell-free extracts of *M. phlei* (of high enzymatic activity) with small amounts of lung extracts from normal and infected mice (0.1 ml of extract) for various periods of time and then determined the effect of preincubation on lactic dehydrogenase activity of *M. phlei* by adding lactate and tetrazolium. The results of a typical experiment are shown in Fig. 1. At zero time, lung extract from normal mice stimulated dehydrogenase activity of *M. phlei* extracts by approximately 30%. Preincubation of lung extracts from normal mice with cell-free extracts of *M. phlei* resulted in a gradual decrease in lactic dehydrogenase activity of the latter. After 15 minutes of preincubation lactic dehydrogenase activity of *M. phlei* dropped to control level (cell-free extracts of *M. phlei* without lung extract). Further preincubation brought about an inhibition of lactic dehydrogenase which amounted to 63% after 1 hour. On the other hand, lung extract from infected mice inhibited lactic dehydrogenase of *M. phlei* by 31% at zero time. Preincubation for 15 and 25 minutes resulted in 86 and 90% of inhibition respectively.

Presence of inhibitor in various organs

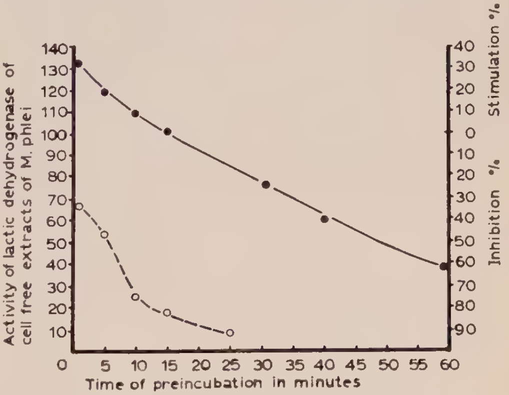

FIG. 1. Effect of preincubation with lung extracts from normal and infected mice on lactic acid dehydrogenase of cell-free extracts of *M. phlei*. Reaction mixture contained: 7 mg protein of bacterial extracts, 0.5 ml; lung extract, 0.1 ml; 0.1M phosphate buffer, pH 7.2, 0.5 ml; 0.3M sodium lactate, 0.1 ml; tetrazolium 1.0%, 0.1 ml. The reaction was stopped after 10 min. of incubation at 37°. Solid line (——), preincubation with lung extract from normal mice. Dotted line (– – –), preincubation with lung extract from infected mice.

TABLE III. Presence of Inhibitor of Lactic Dehydrogenase of *M. phlei* in Various Organs of Normal and Infected Mice.

Cell-free extracts of *M. phlei* in presence of	Reduction of tetrazolium by cell-free extracts of *M. phlei* O.D. of formazan			
	Without pre-incubation	With pre-incubation	Without pre-incubation	With pre-incubation
	Exp. I		Exp. II	
Control without lung extracts	.90	1.10	.98	1.20
Lung extract from infected mice	.32		.30	
Idem normal mice	1.20	.25	1.00	.12
Kidney extract from infected mice	.66		.90	.18
Idem normal mice	1.60	.40	1.75	.32
Liver extract from infected mice	.90	.32	.46	
Idem normal mice	1.60	.42	2.00	.28
Spleen extract from infected mice	.16		.08	
Idem normal mice	.50		.24	

Extracts of various organs were used in 0.25 ml amounts; for other conditions see Table I.

from normal and infected mice. In the light of the above findings it was of interest to examine various organs from both normal and infected mice for presence of the inhibitor. The results of 2 typical experiments (Table III) show that extracts from organs of normal and infected mice inhibit lactic dehydrogenase of *M. phlei* but to a different degree. Inhibitory activity of organs from infected mice was always higher than that of corresponding organs from normal animals. In the majority of cases inhibition of lactic dehydrogenase of *M. phlei* by extracts of organs from infected mice could be readily demonstrated (no preincubation was required). On the other hand, extracts of various organs from normal mice, with the remarkable exception of spleen, inhibited lactic dehydrogenase of *M. phlei* only after preincubation. Spleen extracts both from normal and infected mice inhibited lactic dehydrogenase of *M. phlei* without preincubation. It can be seen however, that the inhibitory activity of spleen extracts from infected mice was always much more pronounced than that from normal animals.

Discussion. It has been previously shown (1,2) that intact cells and cell-free extracts of the *in vivo* grown tubercle bacilli as well as lung extract from mice infected with $H_{37}Rv$ inhibited the electron transport system of various mycobacteria and of lung homogenates of normal mice. These findings posed the question as to the origin of the inhibitor. Experiments reported here show clearly that the inhibitor is present not only in the organs of infected mice but also in those of normal ones, which indicates that the origin of the inhibitor is the animal itself and that the inhibitory activity of tubercle bacilli grown *in vivo* is probably due to adsorption of the inhibitory substance on the surface of the bacillus. A marked difference in inhibitory activity of extracts from normal and infected mice was found. Extracts from mice infected with $H_{37}Rv$ always displayed a much more pronounced inhibitory activity than those from normal animals. It is clear therefore, that tuberculous infection is accompanied by, or causes an increase in inhibitory activity of the infected organs, probably by virtue of the stimulation of production of the inhibitor by tubercle bacilli. It is of great interest to elucidate the possible role of the inhibitor in the normal animal as well as in the tuberculous infected one (and possibly in other infections as well). Concerning the role of the inhibitor in the tuberculous process one may speculate that the inhibitor is introduced into phagocytes upon ingestion of tubercle bacilli coated with this substance. This process may bring the inhibitor in close contact with the respiratory enzymes of the phagocyte, thus causing inhibition of their respiration and eventually their death. Concerning the presence of the inhibitor in organs of normal mice, it would be of interest to determine whether it is in any way involved in regulation of respiratory processes of the tissues. In connection with the role of spleen in the defense mechanism against infections, the presence of relatively large amounts of the inhibitor in this organ is noteworthy. These aspects are presently under investigation.

Summary. The presence of an inhibitor of the electron transport system of a number of mycobacteria and of lung homogenates of normal mice was found in normal mice and in those infected with *M. tuberculosis* $H_{37}Rv$. This inhibitor was previously found in tubercle bacilli grown *in vivo* and in lung extracts from infected mice. This work shows that the amount of inhibitor is greatly increased in animals infected with $H_{37}Rv$. The significance of these findings is discussed.

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Studies of *Mycobacterium tuberculosis* $H_{37}Rv$ Grown *in vivo:* Utilization of Glucose. (26193)

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Segal and Bloch(1) in their study on biochemical differences of *Mycobacterium tuberculosis* $H_{37}Rv$ grown *in vivo* and *in vitro,* compared the respiration of the 2 kinds of cells. They showed that both *in vitro* ($H_{37}Rv$) and *in vivo* ($LH_{37}Rv$) grown organisms had a similar rate of endogenous respiration but differed in their response to exogenous substrates. While respiratory response to glucose and its intermediates by $H_{37}Rv$ cells ranged from 59 to 102% depending on the substrate used, tubercle bacilli separated from lung tissues gave no response to any of these substrates above that of the endogenous. Discussing the failure of the $LH_{37}Rv$ cells to oxidize glucose and its intermediates, these authors suggested that this may be caused either by lack of permeability of $LH_{37}Rv$ cells to glucose or by an over-supply of endogenous food reserves. Although these factors would not readily explain the specific differential response to salycilic acid and fatty acids, neither of these possibilities was ruled out.

Reports from this laboratory(2,3,4) have shown that tubercle bacilli grown *in vivo* inhibit the electron transport system in cell-free preparations of various mycobacteria and in lung homogenates of normal mice. Inhibition of the electron transport system by tubercle bacilli grown *in vivo* is brought about by an as yet unidentified substance which has its origin in the host. This substance upon adsorption on the surface of the tubercle bacilli confers on them the inhibitory activity. In the light of these findings one can also assume that the inhibitor by coating the *invivo* grown tubercle bacilli interferes in some way with uptake and utilization of glucose by these cells. Since glucose is the only sugar present in animal tissues it seemed highly improbable that *in vivo* grown tubercle bacilli would be incapable of utilizing this important source of energy. We attempted, therefore, to answer this question by studying uptake and utilization of C^{14} Glucose by *in vivo* grown tubercle bacilli.

Materials and methods. The organisms used were: *Mycobacterium tuberculosis* $H_{37}Rv$ and *Mycobacterium tuberculosis* $LH_{37}Rv$. *Mycobacterium tuberculosis* $H_{37}Rv$ was grown and maintained in a liquid medium of the following composition: asparagine, 5.0 g; KH_2PO_4, 5.0 g; citric acid, 1.5 g; ferric ammonium citrate, 0.05 g; $MgSO_4$, 0.5 g; K_2SO_4, 0.05 g; glycerol, 0.5 g; Tween-80, 0.5 g; distilled water, 1,000 ml; bovine albumin to a final concentration of 0.25% was added after sterilization. The tubercle bacilli grown *in vivo* ($LH_{37}Rv$) were obtained from lungs of moribund mice infected with *M. tuberculosis* H_{37}Rv. The infection was caused by intravenous injection into white mice of 15-20 g of 0.2 ml of a 6-day-old culture of H_{37}Rv. The cells were separated from the lungs by differential centrifugation described elsewhere(3). For experiments, both the H_{37}Rv and LH_{37}Rv cells after separation from the respective media were washed twice in a 0.1M phosphate buffer, pH 7.2, containing Tween-80 0.05%, and suspended in the same buffer to the desired density as measured in a Coleman Jr. spectrophotometer. A standard curve relating optical density to dry weight of the bacteria was prepared for each strain. Dry weight was obtained by weighing the standard suspensions dried at 105° for 24 hours. In the experiments on uptake of C^{14} glucose, the cells at the end of experiment were centrifuged off, washed and placed on 2 cm^2 aluminum disks, dried, weighed and counted. The counts were corrected to infinite thickness. Oxygen consumption of H_{37}Rv and LH_{37}Rv cells was measured by the conventional Warburg technic. In experiments with C^{14} labeled glucose, the center well contained a 15% solution of CO_2-free KOH, and expired $C^{14}O_2$ was trapped directly into this solution. At the end of experiment, contents of the center well were quantitatively removed into stoppered tubes to which a known amount of carbonate carrier was added. The carbonate was precipitated by dropwise addition with stirring of an excess of 12% $BaCl_2$. The barium carbonate was centrifuged in stoppered tubes and washed several times to remove all traces of alkali. The washed $BaC^{14}O_3$ precipitate was dried *in vacuo,* and the dry powder mounted on disks 0.3 cm^2 and counted at infinite thickness in thin end-window Geiger-Muller tube. Specific radioactivities were obtained by direct comparison with a poly /C^{14}/ methacrylate standard (1 µc/g) obtained from the Radiochemical Centre, Amersham, Bucks. Standard error in all determinations did not exceed 5%. Uniformly labeled /C^{14}/ glucose was obtained from the Radiochemical Centre, Amersham, Bucks.

Results. Uptake of C^{14} glucose by H_{37}Rv and LH_{37}Rv cells. Our preliminary experiments on respiration of *in vivo* and *in vitro* grown tubercle bacilli showed that both H_{37}Rv and LH_{37}Rv cells had a similar rate of endogenous respiration with Qo_2 ranging from 8 to 10. The respiratory response of H_{37}Rv cells to glucose varied from experiment to experiment averaging 30% over that of the endogenous. On the other hand, the response of LH_{37}Rv cells to glucose only occasionally exceeded 5% over the endogenous. These results confirmed the findings of Segal and Bloch(1). To determine whether the low or even complete lack of respiratory response to glucose displayed by LH_{37}Rv cells reflected impermeability of these cells to glucose, we studied the uptake of C^{14} glucose by LH_{37}Rv cells and compared it to that of *in vitro* grown cells. For this purpose, 40 mg of dry weight of H_{37}Rv and LH_{37}Rv cells were added to duplicate 100 ml conical flasks containing C^{14} glucose, phosphate buffer and Tween-80 and the mixture incubated for 30 minutes in a shaker at 37°. At the end of incubation time the cells were removed by centrifugation, washed twice with water and finally measured for radioactivity. The results of a typical experiment are shown in Table I. LH_{37}Rv cells take up C^{14} glucose in considerable amounts, exceeding those taken up by the H_{37}Rv cells. The amount of C^{14} glucose taken up by LH_{37}Rv cells in 30 minutes was 8 µmoles as compared to 5 µmoles taken up by H_{37}Rv cells under identical experimental conditions. These results show clearly that *in vivo* grown tubercle bacilli are readily permeable to glucose and therefore, permeability is not the factor responsible for lack of respiratory response of LH_{37}Rv cells to this substrate.

TABLE I. Uptake of C^{14} Glucose by Non-Proliferating Suspensions of H_{37}Rv and LH_{37}Rv.

	C^{14} glucose added		C^{14} glucose taken up	
Cells	µcuries	µmoles	µcuries	µmoles
H_{37}Rv	8.0	100.0	.40	5.0
LH_{37}Rv	8.0	100.0	.64	8.0

Reaction mixture contained: bacterial cells, 40 mg (dry wt); C^{14} glucose as indicated; 0.1M phosphate buffer pH 7.2, 2.5 ml; Tween-80, 0.05% final conc.; final vol 10 ml; time of incubation 30 min.

Utilization of C^{14} glucose by H_{37}Rv and

TABLE II. Utilization of C^{14} Glucose by $LH_{37}Rv$ and $H_{37}Rv$ Cells.

Cells	Q_{O_2}		Respiration rate, increase over endogenous (%)	Total CO_2 expired (μmoles)		$C^{14}O_2$ (μmoles)	Radioactivity of $C^{14}O_2$ from total
	Endogenous	Glucose		Endogenous	Glucose		
$H_{37}Rv$	9.11	11.30	24.0	8.48	10.50	2.30	22.0
$LH_{37}Rv$	9.50	9.75	2.6	8.48	8.67	3.21	37.0

Each Warburg vessel contained: bacterial cells, 7.0 mg (dry wt); 100 μmoles of phosphate buffer, pH 7.2; 20 μmoles of C^{14} glucose of total radioactivity 1.66 μcuries; 0.2 ml of CO_2-free KOH, 15% sol in center well; final vol 2.2 ml; time of reaction 3 hr.

LH_{37} Rv cells. After we had shown that glucose was readily taken up by the *in vivo* grown cells, we next performed experiments designed to ascertain whether these cells oxidize this C^{14} glucose. In these experiments we determined respiration rate as well as glucose utilization by both $H_{37}Rv$ and $LH_{37}Rv$ cells. For determination of the extent of glucose oxidation, $H_{37}Rv$ and $LH_{37}Rv$ cells were allowed to respire in presence of C^{14} glucose until approximately 10 μmoles of CO_2 were evolved, then radioactivity content of the expired $C^{14}O_2$ was determined. Results of a typical experiment are illustrated in Table II. The Q_{O_2} of the endogenous respiration of both $H_{37}Rv$ and $LH_{37}Rv$ cells was about 9. Presence of C^{14} glucose in the reaction mixture resulted in an increase of respiration rate of $H_{37}Rv$ cells by 24% over that of the endogenous, while the respiratory response of $LH_{37}Rv$ cells to the added glucose amounted only to 2.6% over the endogenous. Radioactivity measurements of the expired CO_2 revealed that in the case of $H_{37}Rv$ cells, 22% of total CO_2 was radioactive. The radioactivity found in CO_2 expired by $LH_{37}Rv$ cells amounted to 37% of the total. Results of this experiment show unequivocally that *in vivo* grown tubercle bacilli oxidize the exogenous glucose to a greater extent than those grown *in vitro*. Moreover, these results indicate that the lack of respiratory response to glucose by *in vivo* grown tubercle bacilli is apparent but not real, being caused by suppression of their endogenous respiration by glucose. It is apparent that the added C^{14} glucose was without effect on endogenous respiration of $H_{37}Rv$ cells, since the amount of glucose utilized could be wholly accounted for by their increased rate of respiration in its presence.

Discussion. The experiments described here were performed to elucidate the mechanism involved in producing the differences in response to glucose by *in vivo* and *in vitro* grown tubercle bacilli. The experiments on uptake and utilization of C^{14} glucose by *in vivo* grown cells have ruled out both the permeability factor and the inability of *in vivo* grown bacteria to oxidize exogenous glucose as possible causes of the lack of respiratory response to glucose. It was assumed therefore, that other factors might be responsible for this effect; *i.e.*, saturation of the respiratory enzymes involved in glucose oxidation by the endogenous substrates or suppression of endogenous respiration of $LH_{37}Rv$ cells by glucose. One way of distinguishing between these two possibilities would be to determine the respiratory response of $LH_{37}Rv$ cells to glucose at different levels of depletion of the endogenous substrates. This approach is however not feasible since Segal and Bloch (1) showed that endogenous respiration of $LH_{37}Rv$ cells was stable over 20 hour period with no response to glucose during that time. Another approach to this problem would be testing the respiration of cell-free extracts of $LH_{37}Rv$ freed of endogenous substrates by dialysis. Unfortunately, this approach seems also impractical since we have shown(2,3) that $LH_{37}Rv$ cells are coated with a respiratory inhibitor. Disintegration of $LH_{37}Rv$ cells brings this inhibitor in contact with its respiratory enzymes, thus causing a complete abolishment of any electron transfer activity that may have been present in $LH_{37}Rv$ extracts. The results of these experiments also indicated that the inhibitor of the electron transport system, present on the cell surface of *in vivo* grown tubercle bacilli does not interfere with glucose uptake or its utilization by these cells.

Summary. It was shown that *in vivo* grown tubercle bacilli are readily permeable to glucose and oxidize it to an even greater extent than those grown *in vitro*. It was therefore concluded that the apparent lack of respiratory response to glucose by *in vivo* grown tubercle bacilli was not due to either permeability factor or to inability of these cells to oxidize exogenous glucose. Possible causes for the apparent lack of respiratory response of $LH_{37}Rv$ cells to glucose are discussed.

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Cultivation of Two Strains of Killer *Paramecium aurelia* in Axenic Medium.* (26194)

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Certain strains of *Paramecium* contain particles, kappa, in their cytoplasm which are essentially independent genetic units capable of undergoing mutation and self-reproduction. Kappa-bearing paramecia (killers) are capable of killing other particle-free paramecia (sensitives) and are resistant to the action of a toxic agent which they produce(1).

As pointed out by Stock, *et al.*(2), destruction of kappa by chemical means without injury to the host indicates a degree of selectivity on the part of the agent tested. Of over 70 purines and pyrimidines tested by these workers, only 2-6 diaminopurine was found to reduce the kappa content of killers; certain antibiotics have also been reported to have this effect(3). The foregoing experiments were carried out with bacterized cultures. Hence, the role played by these bacteria cannot be adequately evaluated and the need for axenic (bacteria-free) cultures is emphasized.

Reexamination of the kappa system in axenic medium for its possible application to screening potential anti-cancer agents forms the basis of a future investigation. As a preliminary step this paper reports successful development of an axenic medium for 2 killer stocks of *Paramecium aurelia*.

Materials and methods. 1) *Stock cultures.* Killer stocks 51 and 299 of *Paramecium aurelia* and a corresponding sensitive strain of stock 51 were used. Stock 299, a recently discovered killer, contains particles somewhat larger than kappa, called lambda(4). Cultures were obtained from Dr. T. M. Sonneborn of Indiana University, and maintained at 27°C in a 0.15% infusion of Cerophyl (Cerophyl Labs, Kansas City, Mo.) previously inoculated (1-2 days) with *Aerobacter aerogenes;* transfers were made at intervals of 48 hours. In this manner the organisms were maintained at approximately one fission per day and kappa content ranged from 200 to 500 particles per cell. Mass cultures were prepared by doubling the volume with Cerophyl infusion until approximately 1 to 2 l of culture were obtained. Population densities approximated 500-1000 animals per ml. 2) *Sterilization of killer paramecia.* After a number of attempts at sterilization using antibiotics in combination with several washing procedures, the following method gave satisfactory results. Mass cultures of the ciliates (usually 1 or 2 l) were twice filtered through cotton to remove accumulated debris, concen-

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* Work done under Government contract to the CCNSC, Nat. Cancer Inst., N.I.H. The author wishes to thank Mrs. Rae Woods for technical assistance.

trated to a small volume by filtration through Whatman No. 1 paper, and placed in an apparatus designed by Claff(5) for sterilization of certain protozoa. The technic is essentially a washing procedure which takes advantage of the fact that paramecia are negatively geotropic. To enable the ciliates to digest bacteria present in vacuoles, a modification was made which allowed a stationary period of 5 hours. Neomycin (50 μg/ml) was added to the wash fluid. As a test for sterility, individual paramecia obtained by this method were plated on nutrient agar. 3) *Aceto-orcein stain for kappa.* Available technics for visualization of kappa (Feulgen and Geimsa Stains) were time-consuming. A simpler and more rapid method was developed using aceto-orcein.

The dye was prepared by refluxing 1 g of orcein (Matheson, Coleman, and Bell) in 100 ml 50% acetic-acid, cooling to room temperature and filtering through Whatman No. 1 paper. One or more animals were added to a small drop of the dye on a glass slide by means of a micropipette. A cover slip was placed over the drop so that the organisms could be observed under oil immersion. Kappa and the nuclear apparatus stained dark red against a light background. 4) *Method of assay of test culture media.* The technics for handling paramecia were essentially those described by Sonneborn(6). Individual animals were picked up in micropipettes (bore size = 150 to 200 μ) equipped with a rubber bulb on one end, and placed in a volume of test culture fluid. In practice, humidifying chambers were prepared from sterile Petri dishes (25 mm x 150 mm dia) containing three, 3-spot glass depression slides by addition of approximately 20 ml of sterile water to the bottom plate. A volume of 0.3 ml of test culture medium was added to each depression and inoculated with single sterile paramecia; plates were incubated at 27°C. Routinely, animals were removed aseptically with a sterile micropipette, stained with aceto-orcein and examined for the presence of kappa.

Each medium was tested with 3, 6 or 9 individual paramecia. When populations of 64 to 128 cells were reached (5-6 days), the animals were transferred to tubes containing 4 ml of test media. Surviving tube cultures were tested for sterility in the following media: thioglycollate, nutrient broth, 0.5% yeast extract plus 1% glucose, and 0.3% beef extract. If no bacterial growth was observed after 2 weeks at 27°C, the protozoan cultures were considered sterile.

Results. The ability of bacterized Cerophyl to support the growth of killer animals suggested trial of this medium supplemented with killed bacteria. Consequently, cultures of *Aerobacter aerogenes* were killed by heat, solvents and freeze-thaw procedures and added to Cerophyl medium alone and in combination with various crudes. None of the media tested was effective in supporting the growth of killers. Feeder cultures of living bacteria placed in dialysis sacs and suspended in a variety of culture media were also ineffective in cultivating the ciliates.

A medium consisting of 7 vitamins, a yeast preparation (AIF-*acetone insoluble fraction* of a hot water extract of Fleischman's yeast), proteose-peptone and stigmasterol, which supported the growth of a sensitive strain of *P. aurelia*(51) was available(7,8). This medium supported the growth of killer animals when supplemented with Edamine S (an enzymatic digest of lactalbumin-Sheffield) but killers lost their kappa after 5-8 fissions.

Constituents of the medium were tested, in various concentrations and combinations, for effects upon growth and maintenance of kappa. Increased concentrations of the yeast fraction (AIF) were toxic. Dialysis of the preparation removed some of the toxic material and made possible the testing of larger amounts. Media supplemented with dialysed AIF allowed growth of some clones in which kappa was maintained for 10 to 15 fissions before being lost. A fresh fraction, (NDF - *nondialysable fraction*(9)) prepared by dialysing a hot water extract of Fleischman's yeast against running deionized water and distilled water for 48 hours, was found to be an effective replacement for AIF in supporting the sustained growth of killers and maintenance of kappa in the cells.

Table I shows the final composition of the medium and Table II the essentiality of vari-

TABLE I. Composition of Axenic Medium* (μg/ml Unless Otherwise Specified).

Compound	Conc.	Compound	Conc.
Ca pantothenate	2.5	K_2HPO_4	250.0
Nicotinamide	2.5	KH_2PO_4	250.0
Pyridoxal HCl	2.5	Ethylenediaminetetraacetic acid (disodium salt)	5.0
Riboflavin	2.5	$MgSO_4 \cdot 7H_2O$	25.0
Folic acid	1.25	$Fe(NH_4)_2(SO_4) \cdot 6H_2O$	6.25
Thiamine HCl	7.5	Proteose-peptone	5.0 mg
Biotin	.625 mμg	Edamine S†	10.0 "
Stigmasterol	.2	NDF (yeast fraction)‡	5.0 "
$MnCl_2 \cdot 4H_2O$	.125		
$ZnCl_2$	.015		
$CaCl_2 \cdot 2H_2O$	12.5		
$CuCl_2 \cdot 2H_2O$	1.25		
$FeCl_3 \cdot 6H_2O$	.31		

* Proteose-peptone, Edamine S, salts and stigmasterol may be autoclaved together. NDF and vitamins are added aseptically. pH is 6.9.

† Enzymatic digest of lactalbumin (Sheffield Chemical).

‡ Details of preparation can be found in Miller (8).

ous components. While proteose-peptone is not required for growth of the stock 51 killers, it is necessary for maintenance of kappa. If the NDF fraction of medium No. 8 is replaced with nondialysed AIF, the cells become sensitive. Apparently, in addition to a factor (factors) present in proteose-peptone, an additional factor (factors), either absent or present in small quantities in AIF and present in NDF, is required for maintenance of kappa. Killers of stock 299 may be cultivated without loss of lambda in media containing NDF and Edamine S or proteose-peptone.

Maintenance of stocks. Killers of stock 51 are best grown at 27°C as single cell isolates in depression slide culture where they divide at a rate of slightly more than one fission per day. However, tube cultures have been successfully maintained by transferring small volumes (0.1 to 0.3 ml) to fresh medium at 3-day intervals. Kappa populations have been estimated at several thousand particles per cell; killers exhibit strong killing activity when tested against suitable sensitive strains in axenic medium.

Stock 299 has now been maintained at 27°C in tube cultures for several months. Subcultures are made every 5 days and population densities range from 13,000 to 17,000 cells per ml. Periodic examination of the ciliates reveals the presence of an estimated 500 particles per cell although considerable variation has been noted in cells taken at various stages of the growth cycle.

Discussion. In an attempt to cultivate stock 51 killers in a medium consisting of salts, lemon factor (stigmasterol), NDF and proteose-peptone, van Wagtendonk *et al.*(9) reported that the animals underwent one to 4 fissions and died. They pointed out that their results indicated that the presence of kappa in the cell modified the growth requirements of the killers. The additional requirements of the killers, reflected in the medium reported here (Edamine S, EDTA, and increased amounts of NDF), support this idea.

It is of interest to note the differences in nutritional requirements between the killer stocks. Common to both stock 51 and 299 is the NDF; replacement of NDF by AIF for stock 299 results in loss of lambda and indicates the essentiality of a factor(s) present

TABLE II. Essential Constituents of the Axenic Medium and Maintenance of Particles.

	Components of medium			Growth			Retention of particles	
Medium*	Proteose-peptone	Edamine S	NDF	51 (S)	51 (K)	299 (L)	51 (K)	299 (L)
1				0	0	0		
2	+			0	0	0		
3		+		0	0	0		
4			+	0	0	0		
5	+	+		0	0	0		
6	+		+	+	0	+	†	+
7		+	+	+	+	+	—	+
8	+	+	+	+	+	+	+	+

* Medium consists of salts, vitamins and stigmasterol.

† Kappa lost in Medium No. 7 after 5-8 fissions.

in yeast. Moreover, proteose-peptone and Edamine S, both of which are requirements for growth and maintenance of kappa of stock 51, may be substituted for each other without loss of lambda in stock 299.

The complexity of the growth requirements of killers as they differ from sensitives and the differences in these requirements between stocks serves as an indication of the intricate relationships between these remarkable particles and the cells of the host. More detailed analysis of nutritional requirements of the killers is presently being undertaken, to develop a chemically-defined medium. In this way, greater insight into the nature of the particles themselves as well as identity of the factors responsible for their maintenance may be obtained.

Summary. Two killer stocks of *Paramecium aurelia,* 51 and 299, have been cultivated axenically in a medium consisting of vitamins, a salt mixture, stigmasterol, proteose-peptone, an enzymatic digest of lactalbumin and the nondialysable fraction of a hot water extract of yeast. All components are necessary for growth of the ciliates and for maintenance of the kappa character. Differences in nutritional requirements of the two stocks have been noted. As far as is known, this is the first instance of cultivation of a kappa-bearing strain of *Paramecium* in bacteria-free medium.

Since this paper was submitted for publication, stocks 138 and 139 of *P. aurelia,* containing respectively μ and π particles, have been placed in axenic culture (medium No. 8 as given in Table II). The organisms have been maintained in tube culture for two months without loss of particles.

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Influence of Dietary Protein Levels on Ascorbic Acid Status of the Rat.* (26195)

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There appears to be no report regarding the influence of dietary protein on biosynthesis of ascorbic acid in the rat. Prompted by a chance observation that rats receiving a high protein diet excreted relatively large amounts of ascorbic acid in urine, we investigated the influence of dietary protein on the Vit. C status of the rat. In the first instance, the effect of variations in dietary protein levels was examined.

Materials and methods. Adult male albino rats (Wistar strain) with body weights in the range 180-200 g were employed in all experiments. Animals were distributed according to the randomized block design for the various test treatments. Diets fed had the following composition: Protein (Polson's lactic casein)—according to choice; fat (refined peanut oil)—10%; vitaminized starch—1%; salt mixture‡—2%; and corn starch—to 100.

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* A preliminary report of this work was presented at the Symposium on Proteins held at this Institute Aug. 14-16, 1960.

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‡ Hubbell, Mendel and Wakeman mixture.

The vitaminized starch contained thiamine hydrochloride 40 mg, riboflavin 80 mg, niacin 100 mg, pyridoxine hydrochloride 40 mg, calcium pantothenate 100 mg and folic acid 5 mg per 100 g. The rats were given necessary amounts of diluted shark liver oil twice a week to provide the requirements of Vit. A and D.

Cages with wide mesh wire bottoms which were well covered with resistant paint were used to house the animals individually. Urine was collected in brown glass bottles containing 10% trichloracetic acid (TCA) over 3-day periods. Collecting funnels and bottoms of cages were rinsed with 10% TCA solution at least thrice a day during the collection period. Urine collections were made up to known volume and aliquots used for estimation of total Vit. C (ascorbic acid, dehydroascorbic acid and diketogulonic acid) according to the dinitrophenylhydrazine (DNPH) method of Roe and Keuther(1). The same procedure was employed for estimation of ascorbic acid in tissues and blood.

TABLE I. Urinary Ascorbic Acid Excretion of Rats Transferred from Stock Ration to Synthetic Casein Diets. Six rats in each group. Period of feeding, 3 weeks.

Group	% casein in diet	Avg urinary ascorbic acid excretion, mg/day	
I	10	1.18	±.19 (15 d.f.)
II	20	1.82	
III	30	2.10	
IV	50	2.87	

Statistical significance I ∼ II * I ∼ III***
II ∼ III N.S. II ∼ IV **
III ∼ IV *

Differences significant at: *5%, **1% and ***0.1% level. N.S., not significant.

To ensure that the above photometric procedure did not include an appreciable proportion of chromogenic derivatives other than those related to ascorbic acid, DNPH derivatives were prepared(2) from the urine as well as the tissues of test animals (especially in the case of those giving high excretions) and their chromatographic behaviour and absorption spectra studied. On paper chromatograms developed with a number of solvents the derivatives from urine as well as the tissues gave only a single pink coloured band or spot identical in position and absorption characteristics with the derivative prepared from standard L-ascorbic acid (Merck). Also, the derivatives in 12.5 M sulphuric acid solution gave absorption spectra identical with that of the standard derivative under similar conditions (in the range 310-600 mμ, determined with the Beckman spectrophotometer, Model DU).

Results. Effect of protein quantity. Stock rats were transferred to diets containing 10, 20, 30 and 50% casein. After being fed these rations for 3 weeks their urinary ascorbic acid excretion was measured (Table I). There was a progressive increase in excretion of ascorbic acid as protein content of the diet increased; rats on the 50% casein diet excreted more than twice as much ascorbic acid in the urine as rats on the 10% protein ration.

Effect of protein depletion-repletion. Rats were fed a protein-free diet for 2 weeks and urinary ascorbic acid excretions were measured. They were then distributed into appropriate groups and fed diets containing 10, 30 and 50% casein for 2 weeks and their urinary excretion and tissue levels of the vitamin were

TABLE II. Urinary Excretion and Tissue Levels of Ascorbic Acid in Rats Maintained for 2 Weeks on Protein-free Diet and Re-fed Casein Diets for 2 Weeks. Five rats in each group.

Group	Diet	Urinary ascorbic acid excretion, mg/day		Liver ascorbic acid (total), mg		Adrenal ascorbic acid (total), μg		Blood ascorbic acid levels, mg/100 ml	
I	Protein-free diet	.48 ±.12 (4 d.f.)		—		—		—	
II	Re-fed 10% casein diet	1.28	±.30 (8 d.f.)	2.06	±.15 (8 d.f.)	114.8	±6.9 (8 d.f.)	.78	±.07 (7 d.f.)
III	" 30% " "	2.44		4.46		153.0		1.40	
IV	" 50% " "	2.92		4.82		189.0		1.42	
Statistical significance		I ∼ II * II ∼ III* II ∼ IV ** III ∼ IV N.S.		II ∼ III*** II ∼ IV *** III ∼ IV N.S.		II ∼ III** II ∼ IV *** III ∼ IV **		II ∼ III*** II ∼ IV *** III ∼ IV N.S.	

Differences significant at: *5%, **1% and ***0.1% level. N.S., not significant.

TABLE III. Effect of Sulfaguanidine on Urinary Excretion and Tissue Levels of Ascorbic Acid in Rats. Ten rats in each group.

		Urinary ascorbic acid excretion, mg/day				Liver ascorbic acid avg						
Group	Diet	5 wk feeding		10 wk feeding		Total, mg		Liver conc., mg/g		Adrenal ascorbic acid avg, μg		Blood ascorbic‡ acid avg, mg/100 ml
I	10% casein diet	1.6	†			3.38	†	.38	†	96.0	†	1.24
II	*Idem*, with 2% sulfaguanidine	.35	***			1.46	***	.25	***	95.7	***	.76
III	50% casein diet	1.5	±.16	1.5	±.14	2.7	(8 d.f.)	.38	(8 d.f.)	113.4	N.S.	1.2
IV	*Idem*, with 2% sulfaguanidine	1.1	(9 d.f.) N.S.	1.3	(9 d.f.) N.S.	2.4	N.S.	.37	N.S.	108.4		1.4

† As range of variation of values in each group was proportional to the average, logarithmic transformations have been used for purposes of statistical evaluation.

In group I one rat died in the 4th wk. In group II adrenal ascorbic acid was extremely low in one rat. In group III adrenal ascorbic acid value was very low in one case and in group IV one of the rats died during the experiment; for one rat, adrenal sample was lost. Corresponding values for statistical analysis were estimated by the missing plot method (*Experimental Designs* by W. G. Cochrane and G. M. Cox, 1950, pp. 99-100).

‡ Not statistically evaluated as 2 or 3 specimens in each group were lost or could not be obtained.

Statistical significance: *5%, **1% and ***0.1% level. N.S., not significant.

determined. Again, progressive increase in urinary excretions of ascorbic acid with increase in protein content of the diet was evident (Table II). Significant changes in liver, adrenal and blood ascorbic acid levels parallelling urinary excretions were also observed.

Effect of sulfaguanidine. In view of the findings of Schwartz and Williams(3-5) and Schwartz *et al.*(6), that ingestion of antibacterial agents like aminopterin and sulfasuxidine along with the diets markedly reduces the liver level and urinary excretion of ascorbic acid in the rat, the effect of including 2% sulfaguanidine in the low (10%) as well as in the high protein (50%) diets was examined (Table III). In the low protein group there was a marked decrease in urinary excretion of rats receiving the sulpha drug at the end of 5 weeks. These rats were sacrificed for tissue Vit. C determination. In the high protein group, on the contrary, there was no significant difference in urinary excretion as between rats fed the sulpha drug and the corresponding controls. Therefore, they were continued on the same diet for 10 weeks when urinary ascorbic acid excretion was again determined. As even on prolonged feeding the sulfa drug did not affect urinary excretion, these animals were then sacrificed for tissue analysis. In subsequent experiments similar results have been obtained with sulfasuxidine.

Discussion. The evidence presented indicates that changes in protein content of the diet markedly influence urinary excretion and tissue levels of ascorbic acid in rats during short-term feeding. Since the increased excretion on high protein diets is not accompanied by any decrease in tissue levels but, on the contrary, by an increase, it is justifiable to infer that increase in amount of protein in the diet favorably influences the biosynthesis of the vitamin presumably by increasing the liver content of the enzymes involved in Vit. C biogenesis or by increased provision of essential precursors. The former appears plausible in view of the well known dependence of liver cytoplasmic proteins upon the plane of protein nutrition in the rat.

As contrasted with short-term experiments, prolonged feeding (over 3 weeks) levels off the differences in urinary excretion and tissue content of the vitamin between rats receiving high and low protein diets. This may involve an adaptive process.

The adverse effects of the sulfa drug in animals receiving low protein diets appear to implicate the intestinal microflora in biosynthesis of Vit. C in the rat. The protective effect of high levels of dietary protein may be interpreted as the effect of excess protein *per se* on the biosynthetic mechanisms or on the precursors or indirectly on the microflora. Further experiments are in progress to test these hypotheses.

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Effect of Premarin on Survival in Men with Myocardial Infarction.* (26196)

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Considerable epidemiological and experimental evidence suggests that estrogens may be antiatherogenic(1-11). However, only 2 clinical trials of the effect of estrogens on survival in men with myocardial infarction have been reported. Oliver and Boyd(12) failed to observe any effect of ethinyl estradiol on survival but Stamler, Pick and Katz in an interim report(13) found suggestive evidence that mixed, conjugated estrogens (Premarin) increased survival. The latter used doses causing manifest feminization and the results lacked statistical significance.

This paper reports results of a continuing study of Premarin in men using small, well tolerated doses, in which survival in the Premarin group is significantly higher than in the controls.

Material and methods. Clinical material consists of men who had recovered from a myocardial infarction in the Los Angeles County General Hospital or the Cedars of Lebanon Hospital, who were ambulatory, cooperative and considered able to attend our clinics regularly, who were willing to take estrogen if prescribed, who had no requirement for hormone therapy (other than insulin or thyroid), and who were free of concomitant illness threatening survival. Acceptability was determined during the first few visits to the clinic, which was staffed entirely by project personnel. Upon acceptance, patients were randomly allocated to control or treatment with Premarin or one of 2 other estrogens (results with these being reported elsewhere(14)). In those receiving Premarin, dosage was regulated solely by tolerance of the individual and in most cases was 1.25 to 2.5 mg daily. These doses usually gave rise, after some months, to minor breast changes that were not objectionable to the patient. In the event of objectionable side effects, dosage was reduced to the point of ready tolerance.

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Reduced libido was an infrequent complaint (15).

Patients were seen in the special atherosclerosis research clinics at 3 to 6-week intervals, usually by the same physician. Autopsies were secured in about 40% of the fatalities and copies of the death certificate in almost all. Since this study is directed to atherosclerosis alone, we have removed from the series all deaths except those reportedly due to arteriosclerotic heart disease. Furthermore, since we have no reason to expect that very brief treatment with these small doses might be effective, we have confined our analysis to those treated with Premarin or placebo a minimum of 75 days.

Life table analyses were utilized(16). It is important to note that a number of patients, after being treated 75 days or more, subsequently left the clinic and discontinued therapy. Some of these were truly lost (Table I) and were dropped at that point from the analysis. Others, however, remained in contact with the study though no longer under treatment: these individuals, having had at least 75 days of treatment, continue to be counted. Finally, it should be noted that this is an analysis of a continuing study. Thus, a follow-up is now possible through the 39th month on those first admitted to the project. A patient beginning treatment Jan. 1, 1960, however, can enter into the analysis for only the first 6 months, the date of analysis being June 1, 1960. At each interval of time, therefore, there are a number of patients whose period of observation has not yet exceeded that period, although they were still alive, and this number must be taken into account under some heading such as "withdrawn alive." It is clear that these individuals have been "withdrawn" simply because their period of observation has not yet passed the stated interval, rather than for any medical reason.

TABLE I. Survival Rates in Men with Myocardial Infarction Receiving No Estrogen or Treated with Premarin for at Least 75 Days, Showing (1) No. of Months from Beginning Treatment, (2) No. at Risk (*i.e.*, Alive) at Beginning of Each Period, (3) No. of Deaths Due to Arteriosclerotic Heart Disease in the Period, (4) % Surviving through Period, (5) No. with Whom All Contact Became Lost in the Period, and (6) No. "Withdrawn Alive" in the Period Because They Had Been under Observation Only This Long as of June 1, 1960.

Mo of therapy	No. at risk	No. of deaths	% surviving	No. lost	No. withdrawn alive
Control group					
3- 6	123	6	95	1	5
6- 9	111	4	91	1	5
9-12	101	4	88	0	3
12-15	94	1	87	0	4
15-18	89	2	85	1	7
18-21	79	3	81	0	7
21-24	69	3	78	0	8
24-27	58	2	75	0	4
27-30	52	1	73	3	2
30-33	46	1	72	1	4
33-36	40	2	68	2	2
36-39	34	0	68	0	8
Premarin group					
3- 6	62	0	100	1	7
6- 9	54	0	100	0	4
9-12	50	1	98	1	2
12-15	46	1	96	0	5
15-18	40	1	93	0	6
18-21	33	1	90	0	8
21-24	24	0	90	0	6
24-27	18	0	90	0	1
27-30	17	1	84	0	2
30-33	14	0	84	0	4
33-36	10	0	84	0	4
36-39	6	0	84	0	3

Results. Table I shows the period in months from beginning of treatment, number of patients alive and at risk at beginning of the period, number of deaths due to arteriosclerotic heart disease during the period, percentage surviving through the period, number "lost" to all follow-up, and number "withdrawn alive" at end of the period. The percentage surviving (Fig. 1) is of course a cumulative figure.[†]

At each 3-month interval from the 3rd thru the 15th month, p value for the difference in survival rates in the 2 groups was .05, except at the 9th month interval where p value was .01. Beyond 15 months, the numbers of cases which have been currently followed for these long periods is not yet sufficient to lend the differences statistical significance. However, the available data tend to suggest that the significant differences found in the first

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[†] Thus, if 80% have survived through period i-l and if 10% of these survivors die in period i, then 10% of 80% die in period i (or 8%) and 80-8% (or 72%) survive thru period i.

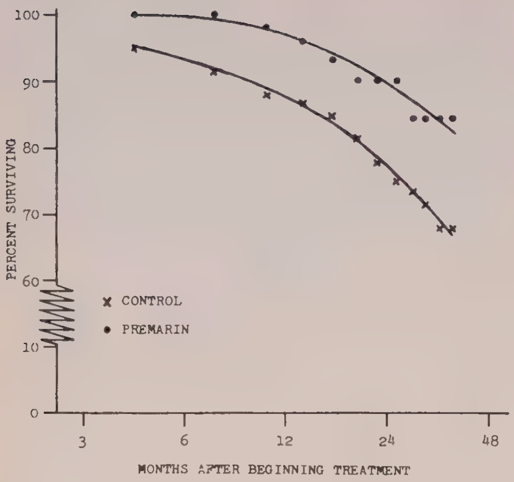

FIG. 1. Graphic representation of percentage survival rates shown in Table I.

15 months may continue or increase thereafter.

Discussion. This is the first demonstration, to our knowledge, of a significant effect of any medical agent on incidence of deaths due to arteriosclerotic heart disease in man. It is of interest that this result was obtained with a mixture of natural estrogens, presumably including a variety of female hormones and their metabolites, administered in doses that failed to cause objectionable evidences of feminization in men. Much of the data (1-10) suggesting that estrogens may be antiatherogenic is based on the epidemiology of coronary artery disease or on the experimental use of Premarin. Our findings are also in accord with the interim report of Stamler, Pick and Katz(13) who used comparatively large doses of Premarin. Our results with this mixture of natural estrogens fail to agree with the negative findings of Oliver and Boyd(12) who used considerable doses of the pure compound, ethinyl estradiol.

Since both Premarin and ethinyl estradiol in sufficient dosage are potent feminizing agents, since one affects survival while the other does not appear to do so, and since our survival effects were obtained with doses that caused only minor breast changes, it seems entirely possible that the beneficial effects of Premarin on survival may be mediated by constituents whose protective activity greatly exceeds their feminizing potency.

Conclusion. The incidence of deaths due to arteriosclerotic heart disease is being compared in men recovered from myocardial infarction and treated 75 days or more with either Premarin or no estrogen (control), allocation of treatments being randomized. Survival rate in those receiving Premarin is significantly higher than in controls.

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Biochemical Changes and Incorporation of P^{32} in Separated Elements of the Hyperplastic Lesion of Fowlpox. (26197)

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Investigation of an animal virus in the medium of the intact host has been superseded in some instances by the more quantitative approach of tissue culture technic utilizing a well-defined cell type. Where feasible, the advantages of tissue culture are conceded, but in the case of fowlpox the virus has not yet been cultivated in an established cell line. The nature of the lesion necessitates investigation in animals since the classical hyperplasia and inclusion body formation are scarcely evident in tissue culture.

It is well known that fowlpox infection involves the skin with conspicuous hyperplasia of the epithelium and thickening of the connective tissue due to hyperplasia and associated inflammatory changes. Concomitant with these events the large conspicuous intracytoplasmic inclusions develop in the epithelial cells. Because of the amount of lipid evident in the inclusion body an investigation of lipid content of whole chick skin and chorioallantoic membrane was undertaken(1). It appeared likely that analysis of whole tissue would not properly portray differences that might occur in the several layers of the skin such as the epithelium which is not invaded by inflammatory cells. Methods were devised to separate the skin into epithelial elements and connective tissue. The present experiment describes differences in the biochemistry and incorporation of P^{32} in the separated structures of fowlpox infected skin.

Materials and methods. Virus. The strain of fowlpox and preparation of inoculum used in this study have been documented(1).

Experimental animals and separation of tissue. Day-old cockerels obtained from a local hatchery were inoculated by gently rubbing a 1:5 dilution of stock material on the freshly plucked scalp, resulting in a confluent lesion. After the desired interval of infection, chicks were dispatched with ether and the scalps immediately excised, carefully rinsed in saline, drained, and placed in 1% Bacto trypsin in 0.02 Tris buffer (pH 7.6) for 17 to 20 hours at 4°C. The tissue then was gently rinsed free of trypsin solution with 0.9% NaCl and allowed to drain. The superficial epithelium could now be easily removed, with curved forceps, as a soft paste, and the turgid, greatly enlarged, hyperplastic follicles expressed from the supporting connective tissue by gentle pressure. After they had been rinsed thoroughly in physiological saline to remove debris, all tissues were frozen and lyophilized to constant weight.

Chemical procedures. The dried tissues were extracted exhaustively at room temperature with chloroform-methanol (2:1). The extracts were taken to dryness under nitrogen (N), and the lipids were repeatedly extracted from the residue with petroleum ether (B.P. 30-60°C). After evaporation under N total lipids were dried to constant weight and redissolved in a known volume of petroleum ether for isotopic and chemical analysis. The methods for performing the various chemical procedures have been noted previously(1), and only the lipid fractions that showed statistical variation as the result of infection were employed in the present study. The possibility that the extracted phospholipid was contaminated with highly radioactive inorganic P^{32} was investigated. Exhaustive experiments in this laboratory reveal that no detectable inorganic P^{32} can be carried over into petroleum ether extracts.

Isotopic procedures. The experiment utilized inorganic P^{32} as $H_3P^{32}O_4$ in HCl solution obtained from Oak Ridge National Laboratory. Quantitative dilutions were made with 0.9% NaCl containing 5 mg of P as

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TABLE I. Lipid Composition of Separated Components of Normal and Infected Chick Skin.*

Tissue	Total lipid† Norm.	Total lipid† Inf.	Phospholipid Norm.	Phospholipid Inf.	Total cholesterol Norm.	Total cholesterol Inf.	Free cholesterol Norm.	Free cholesterol Inf.	Esterified cholesterol Norm.	Esterified cholesterol Inf.
Epithelium										
3 days	39.6	38.8	7.8	14.2	4.0	4.8	N.S.	3.3	N.S.	1.5
5 "	38.8	35.6	6.2	15.7	3.6	8.6	N.S.	4.2	N.S.	4.4
7 "	40.4	33.5	6.3	15.8	3.1	13.5	1.6	5.3	1.5	8.2
Follicles										
7 days		29.3		22.6		17.1		5.9		11.2
Connective tissue										
3 days	29.0	23.3	19.7	26.7	12.2	16.1	6.1	8.8	6.0	7.3
5 "	32.0	14.5	15.9	38.7	9.2	24.1	4.8	14.8	4.4	9.4
7 "	30.7	17.6	17.2	46.4	9.0	18.8	4.4	13.1	4.7	5.7

* Lipid fractions expressed as % of total extractable lipid.
† Total lipid expressed as % of dry tissue wt.
N.S. = Not sufficient material for analysis.

$PO_4(H_3PO_4)$ per liter, as recommended by Zilversmit(2).

Each chick was given approximately 15 μc of P^{32} intramuscularly, and samples of appropriate dilutions of the inoculum were evaporated to dryness on nickel-plated, one-inch diameter planchets, the bottoms of which were lined with 3-mm Whatman paper. (The paper assured uniform distribution of the lipid samples which were to be counted under the same conditions.) The inoculum planchets served as a check on the day-to-day variations in counting efficiency as well as a measure of the isotope given each chick. Activity was determined with a Tracerlab End-Window Geiger Tube and Superscaler. Counts were made to a probable error of ± 1% and corrected for decay according to the widely used expression $A_0 = A/e^{-kt}$ Lipid samples were counted in triplicate in amounts less than 50 mg per planchet. It is pertinent that this amount of inactive lipid could be added to a previously counted inoculum planchet without evident loss of activity due to absorption(3). As the result of preliminary uptake studies it had been determined that maximum incorporation of P^{32} in the skin of the chick occurred 48 hours after administration and that administration of isotope caused no overt changes in lipid composition of the tissues under study. Accordingly, in subsequent experiments all chicks were harvested 48 hours after injection of isotope. For each of the several trials 90 infected and 90 controls received isotope 48 hours before sacrifice. Thirty chicks in each group were killed at 3, 5, and 7 days.

The 3-day period was arbitrarily chosen as the starting point since the skin at this time shows the earliest evidence of infection, and subsequently the lesion develops rather rapidly. The various periods here refer to duration of the experiment and not to age of the chick which was, on the average, 24 hours old at the onset.

Results. Lipid changes. It has already been stated that P^{32} administration did not produce demonstrable isotopic effect on the lipid chemistry of intact normal chick skin.

The lipid changes during course of infection are better compared with the normal when the skin is separated into connective tissue and epithelial elements, *i.e.,* surface epithelium and follicles. These differences are depicted in Table I and represent the average of triplicate analysis of 2 different trials for each time interval. It should be noted that as a group there is a distinct difference between normal epithelium and connective tissue in each of the categories. A comparison of infected epithelium and connective tissue with its normal counterpart shows approximately a 2- to 3-fold or greater increase in phospholipid and total, free, and esterified cholesterol at some time during infection.

It should be noted that during infection one of the components (epithelial phospholipid) did not increase, having reached the

TABLE II. Specific Activities of Separated Layers of Normal and Infected Tissues.*

	Epithelium		Connective tissue		
Hr†	Norm.	Inf.	Norm.	Inf.	Follicles
72	.74	.66	.56	.64	
72	.71	.77	.56	.67	
120	.36	.59	.35	.58	
120	.33	.64	.33	.55	
168	.30	.57	.27	.47	.48
168	.32	.53	.25	.47	.48

* Specific activity =

$$\frac{\text{CPM/mg P}}{\text{CPM/chick}} \times 100 = \frac{\%\text{ inj. P}^{32}}{\text{mg phospholipid P}}.$$

† Hr represent time of sacrifice of chicks and, where pertinent, post-inoculation of virus. Each group received isotope 48 hr before death.

maximum value earlier in development of the lesion. The data concerning the follicles require some comment. The study of these structures is limited to the 7-day period since it is difficult to obtain them in any quantity before this time. Furthermore, comparison with the normal follicle is not possible since these structures cannot be separated from the surrounding tissue. Since the infected follicle is largely composed of hyperplastic epithelium, one might expect the lipid categories to be similar to those of the surface epithelium. With the exception of free cholesterol all of the values are appreciably higher. Finally, for all categories the sum of the components does not approximate 100%. The difference is probably due to free and total fatty acids(1).

Effect of infection on incorporation of P^{32}. A comparison of specific activities (SA) of phospholipid P in the several epithelial structures and connective tissue at different time intervals is shown in Table II. A comparison of SA of normal epithelium and connective tissue at 72 hours reveals that incorporation of P^{32} is about 20% less in the latter. In the older chicks SA of both normal tissues drops sharply, approximately 50% in 7 days. Average specific activity of infected tissues at 72 hours is approximately equal to that of the normal counterpart. However, at 5 and 7 days average incorporation drops slightly and consistently in connective tissue and to a lesser degree in surface epithelium, and in any case is approximately 1.5 to 1.7 times that of the normal controls. It is somewhat surprising that surface epithelium and follicular epithelium have different specific activities. Follicular epithelium and connective tissue exhibit the same activity.

Discussion. It has previously been remarked that administration of P^{32} did not result in a demonstrable isotopic effect so far as the lipid components determined here are concerned. Although the significance is not apparent it is worth noting that the phospholipid content did not change appreciably in noninfected whole skin or in the separated elements, whereas specific activity of the phospholipid phosphorus of normal epithelium and connective tissue was reduced by approximately 50% at 5 and 7 days.

It has been shown here that, as a result of infection, cholesterol components and phospholipid increased percentage-wise in both epithelium and connective tissue, though total lipid of the epithelium did not vary significantly and was considerably decreased in the connective tissue (Table I). The refined data did not include weight of the tissue from which the lipid was extracted. Concerning this relation, the ratio of tissue weight/lipid weight averaged 2.5 for normal epithelium and 2.9 for infected, at 5 and 7 days. On the other hand, weight of infected connective tissue increased considerably over amount of lipid as ratios of normal to infected changed from 3.2 to 6.3. Other than hyperplasia as a contributing cause of added weight of connective tissue, consideration must be given to the contribution made by inflammatory cells which are quite abundant. Of course, a class of compound other than lipid could contribute materially to the added weight.

The apparent increase in lipid phosphorylation reported here as a result of virus infection is, of course, not new. Miroff *et al.*(4) demonstrated a significant increase in P^{32} incorporation into the phospholipids of HeLa cells infected with poliovirus. Moldave(5) found that mouse brain phospholipid has a higher specific activity as a consequence of infection with Theiler's GD VII virus, 1.3 times that of the controls. Infection of KB cells with adenovirus also stimulated uptake of P^{32} into lipid fractions(6). There appears to be no explanation for the increase or disposition of phospholipid molecules during infection. The matter is further complicated by reason of extensive hyperplasia concomitant with virus replication. In this regard Levin(7) cited previous experiments indicating that phospholipid metabolism is stimulated during certain conditions associated with hyperplasia.

The decreasing specific activity noted in noninfected epithelium and connective tissue might be due either to lack of uptake or to an increased turnover rate. On the other hand, the elevated specific activity (gradually decreasing with time) of infected tissues could be due to an increased synthesis of phospholipid compounds or a normal rate of synthesis with deposition in areas of compounds where there is little turnover.

Summary. Normal control and fowlpox-infected chick scalp were separated by trypsin into connective tissue and epithelium. By appropriate technics the infected epithelial tissue was separated into surface epithelium and follicles. Comparison was made between normal epithelium and connective tissue and between normal and infected tissues with respect to certain lipids and incorporation of P^{32} into phospholipid. Distinct differences in phospholipid and total, free, and esterified cholesterol were found between normal epithelium and connective tissue. The above components were increased 2- to 3-fold at some interval of time in both infected epithelium and connective tissue. The specific activities of phospholipid P from all tissues were approximate at 3 days but that of normal epithelium and connective tissue fell approximately 50% at 5 and 7 days. In contrast the incorporation of P^{32} in infected tissues was 1.5 to 1.7 times that of normal controls.

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Measurement of Cortical Binding Capacity in Human Plasma.*† (26198)

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The high levels of cortisol in the plasma of pregnant and estrogen-treated patients have been attributed to an α-globulin named transcortin by Slaunwhite and Sandberg(1) and referred to as corticosteroid binding globulin by Daughaday *et al.*(2). These authors have disagreed on whether binding capacity is elevated during pregnancy(1,2,3). These findings have made pertinent the development of procedures to determine reliably and accurately the binding capacity of plasma.

The protein responsible for binding has not been isolated. The binding process has been studied by ultrafiltration(4), single bag equilibrium dialysis at 4°C(1), "two bag" equilibrium dialysis at 4°C(2), and electrophoresis(5,6). Furthermore, Slaunwhite and Sandberg have indicated that the binding is not readily reversible(1). This has broad implication on the physiologic significance of binding in pregnancy and other conditions.

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‡ Trainee, Nat. Cancer Inst.

Their conclusions, however, were based upon determinations at 4°C, and time required for equilibrium to be achieved at 4°C has not been determined satisfactorily nor is the effect of temperature completely clear.

This report describes a procedure for determination of binding capacity in terms of standard plasma, relates the equilibrium achieved at the various temperatures in an appropriate time interval, and determines the reversibility of binding, thereby clarifying its physiologic significance.

Materials and methods. Rationale of procedure: Without extensive purification of the cortisol binding globulin, knowledge of a number of adsorptive sites, or removal of endogenous hormone already "bound" to the protein, an absolute determination of binding protein concentration cannot be made. Nevertheless, if an unknown quantity of binding protein is compared by dialysis with a standard preparation, a comparative determination can be made.

Accuracy in comparison requires equilibrium with similar specific activities and levels of free cortisol in all compartments. Under these conditions amounts of bound radioactivity represent concentrations of binding protein in accordance with mass action. The procedure reported here utilizes a "2-bag" dialysis technic under conditions permitting complete equilibration of dialyzable components.

Cortisol-4-C^{14} (4.16 μc/mg) was purified by paper chromatography and stored in ethanol at 4°C. Pools of plasma obtained from healthy individuals were diluted 1:5 or as indicated. Seamless viscose cellulose dialysis tubing ⅝ inch in inflated diameter was used in all experiments. Sterile salt-poor human serum albumin was employed as a 1.0% solution in .89% saline which was the diluent in all cases. Penicillin was added to a final concentration of 3,000 units/ml to prevent bacterial action. This antibiotic has been demonstrated not to affect binding(1). When cortisol was added, final ethanol concentration never exceeded 0.25%.

For dialysis, 10 ml of diluted or undiluted plasma was used in each compartment. After dialysis, the contents of each dialysis bag and a 10 ml aliquot of the serum albumin external compartment were extracted 3 times with 1.5 volumes of redistilled methylene chloride. The extracts were evaporated under a fine stream of air and an aliquot of an ethanol solution was plated on tared 3 cm aluminum planchets. Radioactivity was measured at infinite thinness in a Nuclear-Chicago windowless gas flow counter to a probable error of 3%. Recoveries ranged from 81 to 93%.

TABLE I. Effect of Temperature on Equilibration: 72 Hr of Dialysis at Indicated Temperature after 90 Min. of Equilibration of Radioactive Steroid in Original Compartment. Volume changes between compartments were less than 5%.

			Counts/min.	
Temp.	Exp.	Plasma dilution	Original compartment	Added compartment
4°	A	1:2	273	70
	B	1:2	264	44
	C	1:5	230	101
37°	A	1:5	164	171
	B	1:5	161	156

Results. I. Effect of temperature on rate of dialysis (Table I): To permit "complete" adsorption to occur, .05 μg of radioactive cortisol were added to 10 ml of diluted plasma, and kept at 4°C for 90 minutes. A dialysis bag containing an identical amount of plasma was then placed in the vessel and dialysis carried out for 72 hours. Both compartments were extracted and radioactivity content determined.

At 4°C, regardless of plasma dilution, very little cortisol left the original compartment, indicating either that the steroid was firmly bound or that dialysis had not yet reached equilibrium. At 37°C equal amounts of radioactivity were found in each compartment. Clearly, equilibrium was achieved at this temperature and the steroid binding is reversible. II. Rate of dissociation of bound cortisol at 37°: Two plasma samples were obtained from a healthy male before and after 4 days therapy with dexamethasone (.75 mg every 6 hours) given to lower endogenous plasma 17-hydroxycorticoid levels. To 33 ml of the first (normal) plasma was added .5 μg of cortisol-4-C^{14} and this mixture was dialysed against 50 ml of 5% albu-

TABLE II. Diffusion of Cortisol-4-C^{14} from Equilibrated Normal Plasma to Treated Plasma at 37°.

	CPM/10 ml		
Time (hr)	Normal plasma	Treated plasma	Ratio N:T
0	744*	0	—
2	606	66	9.2
4	506	118	4.3
8	465	169	2.7

* 146 cpm unbound. (With similar experiment using 5% serum albumin in each compartment, equilibrium was 95% complete in 2 hr.)

min at 37° for 24 hours. Aliquots of plasma and albumin were then extracted. The remainder of the plasma was divided into three 10.0 ml portions. These were individually dialysed against 10.0 ml portions of the second (treated) plasma for periods of 2, 4 and 8 hours. At these times dialyses were extracted and amount of radioactivity in each compartment measured. Similar experiments done with cortisol in 5% albumin revealed almost complete equilibrium in 2 hours and ruled out the membrane as a limiting factor. The data indicate that dissociation of cortisol from undiluted plasma even at 37° is a slow process with mass action markedly favoring binding. III. Effect of cortisol concentration on binding (Table III): Non-radioactive cortisol was added to 10.0 ml aliquots of diluted pooled plasma. Amounts added simulated increases of 12.5 and 25 μg/100 ml plasma. Altered plasmas were equilibrated in test tubes at room temperature for 8 hours to assure complete binding, then transferred to dialysis bags. These 2 altered plasmas and 2 control 10.0 ml samples in dialysis bags were assembled in a flask containing 80.0 ml of albumin solution and dialysed at 37°C for 18 hours. Next, 0.3 μg of cortisol-4-C^{14} were added to the albumin compartment and dialysis continued at the same temperature for 30 hours. The objective was to determine if the nonradioactive cortisol would irreversibly saturate some of the binding sites or diffuse equally among all the plasma compartments. The experiment was performed in duplicate.

TABLE III. Effect of Simulated Variable Cortisol Levels on Equilibrium of Dialysis at 37°: 8 Hr of Equilibration, 18 Hr Preliminary Dialysis with an Additional 30 Hr after Addition of 0.3 μg of Cortisol-4-C^{14}. Total volumes of plasma compartments extracted were all 10.0 ± .1 ml. HSA compartment equal 80 ml.

	Counts/min.	
Compartment	Exp. 1	Exp. 2
10 ml aliquot HSA	110	120
Plasma (diluted 1:5)	211	314
”	300	328
” + .25 μg F	301	323
” + .5 ” ”	318	lost

Equal binding of radioactive cortisol occurred in all compartments. As expected, the albumin medium contained much less hormone. The implication from this isotopic dilution experiment is that binding is reversible at 37°C and that in spite of the load of simulated endogenous cortisol, relative total binding capacities remained constant. IV. Effect of cortisol concentration and protein concentration on binding (Table IV): To confirm the previous observations and to demonstrate the applicability of the procedure, a third series of experiments was devised. First, 1:5 and 2:5 dilutions of plasma were compared; presumably they would represent approximately 2-fold differences in concentrations of binding protein. To one of the 3 compartments of each system, non-radioactive carrier was added in amounts ranging from 0.5 to 4 μg and representing biologic plasma levels of 12.5 to 50.0 μg/100 ml.

The compartments were equilibrated independently at 37°C for 8 hours, then assembled as described in Table IV and dialyzed for 18 hours at 37°C to establish equilibrium. Finally, 0.1 μg of radioactive cortisol was added to the albumin compartment and dialysis continued for another 30 hours.

It is apparent that the non-radioactive cortisol diffused to all compartments, and that even at 37°C significant binding occurs. In almost all cases the 2:1 binding ratio is present, the 2 low ratios probably reflecting experimental error. Correcting for volume changes, calculated distribution ratio is 1.8 and observed ratio is 1.9 ± 0.3 (mean ± standard deviation). Thus, binding does occur at physiologic temperatures, and equilibrium is obtained regardless of endogenous

TABLE IV. Effect of Variable Cortisol Levels and Binding Protein Content in the System: HSA Compartment 20 ml (10 ml Extracted), Cortisol-4-C^{14} Added to HSA Compartment. Volumes of plasma 1:5 extracted = 10.0 ± .1 ml. Volumes of plasma 2:5 extracted = 11.0 ± .2 ml in all cases. Calculated concentration ratio $= \frac{2.0}{1.1} = 1.8$. Observed ratio = 1.9 ± .3.*

Plasma pool	Exp.	Compartment	mg non-radioactive steroid added	CPM (total)	CPM (bound)	Ratio
I	1	HSA	—	79	—	
		P 2:5	—	348	269	1.9
		P 1:5	.5	218	139	
	2	HSA	—	79	—	
		P 2:5	.5	353	274	2.2
		P 1:5	—	204	125	
	3	HSA	2.0	92	—	
		P 2:5	—	328	227	2.0
		P 1:5	—	212	111	
	4	HSA	4.0	111	—	
		P 2:5	—	278	156	2.1
		P 1:5	—	175	74	
II	1	HSA	—	92	—	
		P 2:5	—	298	197	1.8
		P 1:5	.5	203	112	
	2	HSA	—	97	—	
		P 2:5	.5	252	150	1.5
		P 1:5	—	198	101	
	3	HSA	—	106	—	
		P 2:5	—	265	149	2.0
		P 1:5	1.0	181	74	
	4	HSA	—	100	—	
		P 2:5	1.0	266	156	1.4
		P 1:5	—	210	110	

* Mean ± stand. dev.

plasma levels of cortisol because the protein-steroid complex is dissociable.

Discussion. Two dialysis methods for study of the protein binding system have been published. Slaunwhite and Sandberg (1) employed a single bag of diluted plasma dialyzed at 4°C for 72 hours, performing the experiment in duplicate with one microgram of non-radioactive cortisol in the second system. These conditions do not permit complete equilibrium with similar specific activities in both compartments. The technic tends to measure binding capacity of the unoccupied binding sites. Nevertheless, the dilution of plasma permits some dissociation of the steroid-protein complex, and differences in total binding capacity may be detected.

Daughaday, *et al.*(3) employed a 2 bag dialysis on undiluted plasma at 4°C. If equilibrium is achieved, this system may be employed for determining relative binding capacity of various specimens. But at 4° equilibrium with undiluted plasma is not obtained and observed binding represents for the most part free rather than total binding sites. Endogenous cortisol levels of samples may not be equivalent whereas free sites may be equal. In this situation, a plasma sample may have a total binding capacity greater than the standard but it might not be demonstrable if only the free sites are determined.

An ideal method would determine quantitatively the total number of sites and amount of either bound or free sites. Since the ratio of free and bound sites is not easy to determine, the next preferable procedure would determine number of total binding sites relative to a standard.

The procedure reported here utilizes diluted plasma, dialysis at 37°C for 48 hours and 2 or more bags to permit comparison in terms of a standard plasma. It has been demonstrated that under these conditions dialysis of the free cortisol through the membrane is rapid. Dissociation of the steroid-protein complex, and therefore equilibrium, is achieved. Specific activity in each compartment is equivalent. Then, by direct measuring the radioactivity in each compartment, an accurate determination of relative total binding capacity may be made.

The procedure has practical significance. Sandberg and Slaunwhite(3) reported that diluted pregnancy plasma has a higher binding capacity than normal, but Daughaday's data from undiluted plasma, dialyzed against a control plasma, revealed that number of available binding sites was approximately the same(2). The difference is readily resolved because the former authors were at least partially measuring total capacity while the latter was determining free binding sites almost exclusively. Thus, the higher concentrations of cortisol which occur in pregnancy plasma were occupying some of the available sites in the latter incubations, and the amount of free sites (and possibly free cortisol) was equivalent.

The physiologic significance of the steroid-protein complex remains undetermined. Presumably, the complex may be physiologically inactive; in this case, it might act as a buffer against excessive concentrations of steroid. On the other hand, the slow rate of dissociation *in vitro* would mitigate against its being a reservoir of readily available steroid. It is possible, however, that the organism has a more efficient method of dissociating the complex or that the complex may be effective in certain systems as an entity. Finally, if one considers synthesis of this protein as an artifact of pregnancy (and it has been demonstrated to be elicited by estrogen in both males and females), it may have no "role" whatsoever and the higher levels of corticoids found in the plasma in pregnancy may reflect nothing other than homeostatic response to lack of available unbound steroid.

Summary. Simultaneous dialysis of 2 diluted plasma samples in the same system at 37°C is an acceptable method for measurement of the relative amounts of corticosteroid binding protein present. Dissociation of the steroid-protein complex in undiluted samples is slow even at 37°. The kinetics of binding do not permit attainment of equilibrium at 4°, even when the plasma is diluted. The differences noted by previous investigators in comparison of pregnant and non-pregnant samples are explainable by this incomplete equilibrium.

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Factors Affecting Extractability of Cholesterol from Lyophilized Sera by Cold Chloroform.* (26199)

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The significance of the fraction of serum cholesterol which is extracted when lyophilized serum is shaken with cold *anhydrous* chloroform for 3 hours has been studied in

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our laboratory for some years. The excellent reproducibility of replicates suggests that the technic measures a definite fraction of total cholesterol. The possible relationship between atherosclerosis and high values for this fraction, designated "readily extractable" cholesterol (REC), has been emphasized(1,2). Although concentration of this REC fraction increased in most subjects as total serum cholesterol increased, the marked individual variations suggested that other factors may be involved. We wish now to report on 4 additional factors which affected its concentration: (a) cholesterol and triglyceride content of whole serum and chylomicra ("chylomicra" as used here represents material rising to the surface when serum is centrifuged in a refrigerated centrifuge at 20,000 × g for 2 hours), (b) addition of sodium salts of fatty acids and of bile salts to the serum prior to lyophilization, (c) addition of crystalline human serum albumin to the serum prior to lyophilization, and (d) moisture in the chloroform.

Methods. The "readily extractable" cholesterol (REC) of whole serum was determined by the technic described by Forbes *et al.*(1). Triglycerides of serum and infranate were determined by a modification of a procedure described by Van Handel and Zilversmit(3). Two 3 ml aliquots of the chloroform extract were evaporated, and 0.5 ml of 0.4% alcoholic KOH was added to one tube and 0.5 ml of alcohol to the other, followed by heating at 60-70°C for 30 minutes. Two ml of water and 0.1 ml of 10 N H_2SO_4 were then added, followed by 0.5 ml of saturated KIO_4. After 7 minutes, 0.5 ml of M $NaAsO_2$ was added, and each tube diluted to 10 ml with water. One ml aliquots were transferred, 10 ml chromotropic acid (prepared as described by Van Handel and Zilversmit) added to each, and the solutions heated in a boiling water bath away from direct light for 30 minutes. After cooling, color was read at 570 mμ. Total cholesterol content of serum and infranate were determined by a minor modification of a previously described procedure(4).

Results. Part (a). Effect of cholesterol and triglyceride content of serum and chylomicra: Experimental results are shown in Table I. Considering average serum cholesterol and triglycerides, there was a general tendency for REC concentration to increase as concentration of these 2 fractions in-

TABLE I. Ranges of Triglyceride and Cholesterol Concentrations of Whole Serum and Chylomicra and Whole Serum "Readily Extractable" Cholesterol (REC).

		Whole serum ranges		Chylomicron ranges		
No. of subjects	No. of sera	Cholesterol	Triglyceride	Cholesterol	Triglyceride	Whole serum REC ranges
		mg %				
		Heterogeneous groups* (arranged in order of increasing triglyceridemia)				
51	54	164–362 (275)	51–172 (113)	0–37 (7)	0–65 (21)	14–44 (29)
47	52	169–584 (325)	184–300 (234)	0–68 (15)	0–198 (50)	16–71 (38)
28	31	184–465 (330)	309–492 (373)	0–48 (23)	12–203 (87)	24–336 (72)
9	12	230–765 (531)	539–960 (674)	33–429 (190)	164–495 (274)	59–556 (244)
3	7	385–600 (517)	1100–1460 (1334)	121–385 (196)	492–792 (691)	323–502 (430)
		Familial hyperlipemic subjects				
3	14	263–913 (433)	1280–9630 (3239)	195–865 (384)	1145–9280 (3060)	112–840 (347)
		Nephrotic syndrome subjects				
9	13	312–1019 (606)	100–620 (337)	0–104 (31)	0–105 (31)	165–959 (544)

* Includes normals, subjects with arteriosclerotic heart disease with and without myocardial infarction, as well as a variety of other conditions.

( ), mean values.

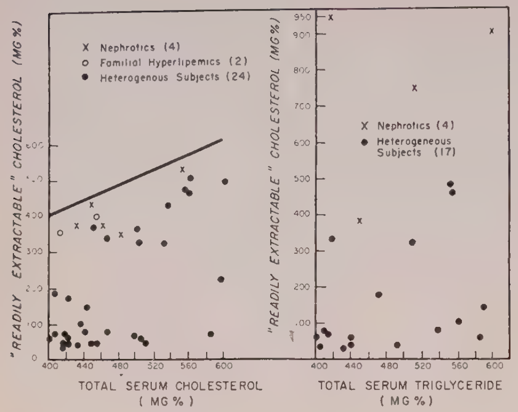

FIG. 1 FIG. 2

''Readily extractable'' cholesterol of serum plotted against total cholesterol (Fig. 1) and triglycerides (Fig. 2).

creased. However, there was such marked individual variability from this generalization that it appeared that factors other than total cholesterol and triglyceride probably affected REC concentration. To emphasize this variability, REC values on sera with total serum cholesterols between 400 and 600 mg% (chosen arbitrarily for illustration) are plotted against total serum cholesterol in Fig. 1. Familial hyperlipemics and nephrotics consistently showed a high percentage of total serum cholesterol in the REC fraction. The heterogeneous group showed wide variability and differences could not be correlated with the disease condition. Similar widespread distribution is shown when REC values are plotted against total serum triglycerides in Fig. 2. Turning attention to chylomicron values, REC concentrations rose in general as the amounts of triglycerides and cholesterol in chylomicra increased. However, it was particularly interesting that, in the singular instance of the nephrotic subjects, REC concentration was consistently very high even though amounts of cholesterol and triglycerides in chylomicra were small. A possible explanation for this finding is presented in the discussion.

Part (b)—Effect of addition of sodium salts of fatty acids and of bile salts: Machebouef and Tayeau(5) showed that addition of soaps to serum increased ether extractability of lipids. We consequently decided to determine what effect, if any, addition of sodium salts of various fatty acids to the serum prior to lyophilization would have on REC values. The effects of sodium cholate and sodium glycocholate were also studied. Experimental results are shown in Table II. Sodium butyrate exerted no definite effect, but with lengthening of the carbon chain, activity became quite evident. Sodium stearolate, even at a concentration of only 0.4%, caused approximately 70% of total serum cholesterol to appear in the REC fraction. Sodium cholate was practically inactive but sodium glycocholate showed some effect. Since commercial sodium glycocholate was used, it was possible that impurities in the preparation may have accounted for the effect noted.

Part (c)—Effect of addition of crystalline human serum albumin: Since addition of sodium salts of long chain fatty acids markedly increased cholesterol extractability, a study was carried out on the effect on REC values of addition of crystalline human serum albumin to the serum prior to lyophilization. If the presence of non-albumin bound free fatty acids in the serum were a factor in producing elevated REC values, then their removal by combination with added albumin would be expected to lower REC concentration. Experimental results are shown in Table III. Albumin caused a very marked drop in REC values of all sera with high REC concentrations. A definite drop was also noted in some sera with low REC con-

TABLE II. Effect of Various Surface Active Agents on ''Readily Extractable'' Cholesterol.

Total choles-terol	Cholesterol extracted from lyophilized sera in presence of various additives				Substance added
	0 mg	2 mg*	5 mg*	10 mg*	
mg %					
255	29	29	27	25	Na butyrate
265	24	42	46	235	Na caprylate
265	24	50	196	243	Na laurate
247	33	167	246	—	Na stearolate
266	32	94	111	186	Stearolic acid
272	24	43	41	57	Na glycocholate
272	24	39	36	32	Na cholate

* mg added/.5 ml of serum.

All sera were incubated for 3 hr at 37°C after addition of the respective agent and before lyophilization. Control tubes were similarly incubated.

TABLE III. Effect of Addition of Albumin to Serum on REC Concentration.

Cholesterol		REC values following addition of different amounts of albumin to serum					
Serum	Chylomicron	0 mg	20 mg*	40 mg*	80 mg*	100 mg*	
mg %		mg %					
279 (247–306)	6	30 (22–38)	26 (20–32)	22 (20–26)	19 (17–20)	16 (13–20)	Avg of 6 sera with normal REC values
357	27	66	37	33	—	—	Sera from various subjects with elevated REC values but excluding those with nephrotic syndrome
465	85	392	—	123	—	—	
475	50	123	—	52	39	—	
467	187	264	—	60	55	55	
391	98	188	97	66	—	—	
858	136	860	—	676	230	117	Nephrotic syndrome
810	0	752	—	76	45	39	” ”

* mg albumin/.5 ml of serum.

( ), range.

centrations but in no case did REC value drop to zero.

Part (d)—Effect of traces of moisture in the chloroform used for REC extraction: The results (Table IV), revealed that moisture (.02% H_2O) exerted no definite effect on REC values of most sera with low REC concentrations, but markedly increased it in sera with high REC concentrations, emphasizing the importance of using anhydrous chloroform. The dried zeolite used for cholesterol and triglyceride determinations is an excellent drying agent for this purpose.

Discussion. In view of the increase in REC obtained when soaps were added to sera with low REC concentrations and the decrease in REC when serum albumin was added to sera with high REC concentrations, it appeared that presence of non-albumin bound free fatty acids markedly affected cholesterol extractability from lyophilized sera. Albumin bound fatty acids apparently played no role.

TABLE IV. Effect of Moist Chloroform on REC Value.

No. of sera	Avg total cholesterol	Avg REC value using	
		Anhydrous chloroform	Chloroform containing .02% water
	mg %		
9	290 (237–394)	28 (21–36)	47* (22–167)
4	444 (359–508)	70 (50–122)	350 (317–378)

* Avg excluding 167 value was only 32.

( ), range.

The fact that most of the cholesterol of the nephrotic sera was "readily extractable" while little of it could be accounted for by chylomicron cholesterol seemed particularly interesting. Shafrir(6) found that concentration of free fatty acids (FFA) in nephrotic sera did not differ significantly from that of normal sera, but because of the low albumin content, only 35.7% (12 sera) was albumin bound as compared with 77.6% in 10 normal sera. In both cases, the remaining FFA was presumably bound to lipoproteins. If one assumes that lipoprotein bound free fatty acids would affect REC concentrations in a manner comparable to addition of long chain fatty acids to normal sera, an elevated REC concentration in nephrotic sera would be expected.

In a few sera from the familial hyperlipemic group the amount of cholesterol present in chylomicra was higher than the REC fraction. We do not believe this to be due to experimental error, although such cannot be excluded. The presence of high density lipoproteins in chylomicra, as suggested by Lindgren *et al.*(7), may be a partial explanation since previous work showed that α_1-lipoproteins contribute little, if any, cholesterol to the REC fraction(8).

The tendency for REC concentration to increase with an increase in triglyceride content of the sera can probably be accounted for in 2 ways: first, an increase in chylomicron cholesterol and second, an effect of lipoprotein bound FFA. Shafrir(6) observed a definite tendency for the percentage of total

FFA bound with albumin to be reduced with an increase in total serum lipids. A concomitant increase in non-albumin bound free fatty acids along with an increase in serum triglycerides in accordance with the results herein presented would be expected to lead to an increase in REC values. The observations that traces of moisture in the chloroform used for extraction increased REC values in sera with high REC concentrations, but seldom in sera with low REC values suggests that the moisture effect may also be related to the amount of non-albumin bound free fatty acids, although we have no direct evidence that such is the case.

Summary. A study has been conducted on factors which affect the "readily extractable" cholesterol concentrations in serum. The results showed that (a) in general, REC values increased as triglyceride and cholesterol contents of the serum and chylomicra increased, but a notable exception was high REC values in nephrotic subjects in whom chylomicron contents of cholesterol and triglyceride were low, (b) addition of the sodium salts of long chain fatty acids to serum prior to lyophilization markedly increased cholesterol extractability, (c) addition of crystalline human serum albumin to sera with high REC concentrations markedly reduced REC values; the albumin also reduced REC concentration in most normal sera but in no case did it completely disappear, (d) traces of moisture in the chloroform used for the extraction had little or no effect on most sera with low REC concentration but markedly increased it in sera with high REC concentration. It is suggested that the concentration of non-albumin bound free fatty acids may play a major role in regulating REC concentrations in various sera.

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Detection of Anti-HeLa Antibodies in Rabbit Antiserum by Indirect Hemagglutination* (26200)

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Production of species-specific antibodies in animals injected with cells of various mammalian cultures has been described(1-4). These antiserums agglutinated erythrocytes of the same animal species(1,2), or were effectively cytotoxic *in vitro* for cells of homologous lines(3,4). These and other findings(5) substantiate the validity of immunologic identification of the species of mammalian cells in continuous culture.

Extreme sensitivity of the antigen-antibody reaction in indirect hemagglutination suggested application of the technic to detection and measurement of antibodies to cultured mammalian cells. This preliminary report describes methodology of an indirect hemagglutination test used for detection of anti-HeLa antibodies in serum of immunized

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rabbits, and describes preparation of a sensitizing antigen from HeLa cells.

Materials and methods. Antigen suspensions were prepared from 4-day-old monolayer cultures of HeLa cells washed repeatedly with 0.85% NaCl solution and scraped from glass with a rubber policeman. Suspended cells were diluted with 0.85% NaCl solution to a concentration of $3x10^6$ cells per ml, disrupted by treatment at 9 Kc per second for 5 minutes in a Raytheon magnetostrictor, and treated with 5% cold trichloracetic acid (TCA) for 30 minutes at 8°C to precipitate protein. After centrifugation, sedimented material was resuspended to original volume in a solution at pH 7.2-7.4 containing equal parts of phosphate-buffer 0.15 M and 0.85% NaCl. Resuspended material was stored for 1 hour at 8°C to extract soluble protein from the sediment, and recentrifuged. The supernate, containing from 30 to 40 μg/ml of protein (Kjeldahl) nitrogen, was used for sensitization of tanned erythrocytes.

Sheep erythrocytes for tanning and sensitization were obtained from sheep blood collected in modified Alsever's solution(6), kept at 8°C for 3 days before use. Erythrocytes were washed with 0.85% NaCl solution and tanned by addition of 1 ml of packed cells to 19 ml of freshly prepared tannic acid (Merck), 1:20,000 (w/v) in 0.85% NaCl solution. Erythrocyte suspension in the tannic acid was incubated at 37°C for 10 minutes with frequent agitation(7), sedimented by centrifugation, washed with 0.85% NaCl solution and diluted in the same solution to a concentration of 2.5% according to packed cell volume. This tanned erythrocyte suspension was mixed with an equal amount of soluble protein obtained from HeLa cells, incubated at room temperature for 20 minutes with occasional agitation, and centrifuged. The antigen-coated erythrocytes so produced were washed once with 0.85% NaCl solution and diluted to a final 0.75% suspension.

Indirect hemagglutination was tested by mixing 0.25 ml of sensitized sheep-erythrocyte suspension with an equal volume of serially 2-fold diluted serum in 13x100 mm round bottomed tubes. After thorough agitation, tubes were incubated at room temperature until erythrocytes in control tubes settled evenly, usually in 90 to 120 minutes. Results were scored according to the pattern of settling cells(7); titers were read as the reciprocal of the highest reactive serum dilution. Incubation of tests overnight at 8°C produced similar results. Test serums were diluted in a 1% solution of bovine albumin (prepared from bovine albumin 30% solution, Armour Laboratories), which did not reduce sensitivity of the test and served as an excellent stabilizer of the tanned erythrocyte suspensions.

Anti-cellular serums were prepared by injection of rabbits with HeLa cells grown in yeast extract medium(8) supplemented with 20% human serum. Cells were washed repeatedly with 0.85% NaCl solution, scraped from glass and made into a suspension containing approximately $2x10^6$ cells per ml, frozen and thawed repeatedly, freed of coarse sediment by centrifugation at 500 x g and administered intravenously to 4 rabbits by marginal ear vein. Four injections per rabbit of 2 ml of cell material were given at 4-day intervals. After 1 week, 1 rabbit was given a fifth injection of $5x10^6$ sonically disrupted HeLa cells in Freund's adjuvant distributed at various body sites subcutaneously and intramuscularly. None of the animals showed anaphylactic reactions. Blood was taken from the animals 10 days after the last injection, and serum was stored at –20°C. Before use, samples of test serums were thawed and inactivated for 20 minutes at 56°C. Control serums were collected from randomly selected uninjected rabbits.

Results. Sensitizing antigens from HeLa cells were prepared and coated on tanned sheep erythrocytes as described. To study the effect of tannic acid on cellular sensitizing antigens, HeLa monolayers also were washed repeatedly with 0.85% NaCl solution, treated with 1:2,000 tannic acid for 10 minutes at 37°C, rewashed once with saline solution, scraped from glass, disrupted by sonic vibration, precipitated with TCA and extracted with buffered saline. Protein nitrogen of sensitizing suspensions of cellular material so prepared varied from 15 to 20 μg/ml.

TABLE I. Agglutination of Human and Coated Sheep Erythrocytes by Rabbit Antisera to HeLa Cells.

Rabbit serum	Hemagglutination titers with		
	Untreated human O, Rh− erythrocytes*	Tanned sheep erythrocytes coated with extract of*	
		Untanned HeLa	Tanned HeLa
Anti-HeLa hyperimmune†	512	8192	64
Anti-HeLa immune rabbit 1	64	512	32
2	64	1024	32
3	32	256	16
Normal rabbit 1	<4	<4	<4
2	<4	<4	<4
3	<4	8	<4
Anti-human‡	<4	<4	<4

* Titers expressed as reciprocals of highest reactive 2-fold serum dilutions.

† Rabbits immunized with washed frozen-thawed HeLa cells (''immune rabbit''); 1 rabbit (''hyperimmune'') also given sonically-disrupted HeLa cells in adjuvant.

‡ Lederle Labs.

Tanned sheep erythrocytes sensitized with these antigen preparations were tested for hemagglutination by a) antisera from 3 rabbits immunized with freeze-thaw disrupted HeLa cells, b) serum from one rabbit so immunized and hyperimmunized with sonically disrupted cells in adjuvant, c) normal rabbit sera, and d) commercially prepared anti-human rabbit serum (Lederle Laboratories). For comparison, these sera were tested also for direct agglutination of human blood-group O, Rh-negative erythrocytes(2). As shown in Table I, serum titers determined by indirect hemagglutination were 8 to 16 times higher than titers determined by direct hemagglutination. In this and other experiments, low titers not greater than 16 occasionally resulted from interaction of sensitized sheep erythrocytes and presumably normal rabbit sera. In all experiments, agglutination was absent in tubes containing sensitized sheep erythrocytes in 1% bovine albumin, or tanned or untanned sheep erythrocytes and normal or immune rabbit serum. Other control tubes containing anti-HeLa rabbit serum and HeLa cell protein-sensitized sheep cells, and anti-human rabbit serum and yeast extract-sensitized sheep cells, likewise showed no agglutination.

Discussion. Agglutination by rabbit antisera to HeLa cells of tanned sheep erythrocytes coated with protein materials extracted from HeLa cells produced satisfactorily high titers ranging from 256 to 8,192, when titers of normal sera did not exceed 16. This indirect hemagglutination test proved considerably more sensitive than direct agglutination of human erythrocytes by rabbit antisera to human cells in continuous culture. Tanned sheep erythrocytes treated with yeast extract medium used for routine cultivation of HeLa cells were not agglutinated by anti-HeLa serums, and tanned erythrocytes sensitized with HeLa-cell material were not agglutinated by anti-human rabbit serum, indicating no contribution by the medium to the hemagglutination reaction.

Early experiments indicated that simple disruption of cells by sonic vibration did not yield an antigen adequate for production of reactive coated tanned erythrocytes. This failure was considered to result from large amounts of lipid material released by cellular disruption, which interfered with adsorption of protein antigen by tanned erythrocytes. No success followed ether-chloroform extraction of lipid succeeded by alcohol-precipitation of protein. A highly reactive antigen for coating tanned erythrocytes was obtained by the procedure described herein. Protein nature of this reactive antigen preparation was indicated by its incapacity to sensitize untreated sheep erythrocytes. The preliminary studies reported here indicate that tannic-acid treatment of mammalian cells may alter cellular antigenic constitution, as reflected by partial loss of reactivity of material extracted from tannic-acid treated HeLa cells and reduction in the concentration of protein nitrogen of such preparations. These findings are of interest in relation to the work of such investigators as Brading (9) who reported reduction in agglutinability of tannic-acid treated erythrocytes.

In experiments not reported here, human esophageal epithelial cells of normal origin in continuous culture (Minn. EE 55-12-1) yielded antigen preparations as reactive as those from HeLa cells. Soluble antigen obtained from EE cells reacted strongly with anti-HeLa rabbit serum in the indirect hemagglutination test. These investigations were conducted to see whether indirect hemagglutination offered advantages in sensitivity over direct hemagglutination which might be useful for future approaches to the problem of immunologic identification within species of cells in continuous culture.

Summary. Material obtained from sonically disrupted HeLa cells by extraction of trichloracetic-acid precipitate with buffered saline was used to sensitize tanned sheep erythrocytes. Sensitized erythrocytes were agglutinated to high titer by anti-HeLa rabbit serum, but minimally by normal rabbit serum. Reactivity of the sensitizing extract was considerably reduced by treatment of HeLa cells with tannic acid prior to disruption and extraction.

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Infectious Agents in Infant Diarrhea: I. A Hemagglutination-Inhibition Procedure for Detection of Bacterial Fractions in Infant Sera. (26201)

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Hemagglutination (HA) procedures have been widely utilized since the technic was first described by Keogh *et al.*(1). Modifications of this technic using lipopolysaccharide antigens have been found to be superior to agglutination reactions for detecting antibody titer rises in cases of shigellosis and colienteritis(2,3). Landy and Trapani(4), however, reported that trace amounts of unbound antigen greatly inhibit the hemagglutination test. In some diseases, such as severe pneumococcal pneumonia(5) and typhoid fever(6) circulating antigen has been demonstrated in the blood by means of a precipitin test. The extremely small amounts of antigen required to inhibit the hemagglutination reaction suggested that this phenomenon might provide a basis for an even more sensitive procedure. Indeed Noyes *et al.*(7) were able to detect bacterial endotoxins in the plasma of dogs with experimental peritonitis utilizing a technic based on this suggestion. The present report describes a hemagglutination-inhibition (HI) test developed along similar lines. With this technic it was shown that sera from infants with diarrhea often contain inhibitors that are antigenically related to bacterial strains isolated from their fecal flora.

*Method.** *Preparation of normal human erythrocytes* (*NHE*). Human O Rh negative blood is diluted with an equal volume of Alsever's solution(8) and allowed to equilibrate for several days before use. The red blood cells can be used until evidence of spontaneous hemolysis is observed, usually a period of 4 to 5 weeks. Just before use the NHE are washed 3 times with 3 volumes of triethanolamine buffered saline (TBS)(9) and packed by centrifuging for 3 minutes at

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* This method was developed from information presented by Landy, M., Trapani, R. J., Clark, W. R., Walter Reed Army Inst. of Research Rep., 1955, (WRAIR-56-55).

TABLE I. Standardization of *Shigella flexneri* 2a HA Antigen Lot #30.

Antigen dilution	Final dilution of hyperimmune rabbit serum									NHE	Titer
	80	160	320	640	1280	2560	5120	10240	20480		
1:5	+	+	+	+	+	+	+	+	+	—	20480+
1:10*	+	+	+	+	+	+	+	+	±		10240
1:20	+	+	+	+	+	+	+	±	±		5120
1:40	+	+	+	+	+	+	±	±	—		2560
1:80	+	+	+	+	+	±	±	—	—		1280

* The antigen dilution of choice is 1:10.

+, positive reaction; —, negative reaction; ±, incomplete agglutination, not considered positive reaction.

1800 rpm. A 10% suspension prepared in TBS is used for antigen sensitization.

Production of sensitizing antigen. Smooth colonies, selected on the basis of colony appearance, uniform suspension on growth in broth and lack of autoagglutination on heating in physiological saline at 100°C for 1 hour, are inoculated heavily onto freshly prepared plates of trypticase soy agar containing 5% glycerine. The plates are incubated under 5% CO_2 at 37°C for 18 to 24 hours. The growth is washed off in small amounts of physiological saline and an equal quantity of 0.1N NaOH is added to the harvest. The mixture is heated in a water bath for 60 minutes at 100°C, adjusted to pH 7.0 with HCl and dialyzed in the cold against distilled water for 24 hours using several water changes of approximately 100 volumes each. The dialysant is centrifuged at 1800 rpm for 1 hour and the supernate Seitz filtered. The resulting water clear lipopolysaccharide antigen may be standardized without further treatment or lyophilized and standardized on a dry weight basis.

Standardization of lipopolysaccharide antigen. The highest dilution of lipopolysaccharide giving an HA titer of 2560 to 10240 with hyper-immune rabbit sera prepared according to the method recommended by Edwards & Ewing(10) is determined next. Two-fold dilutions (1:5-1:80) of the liquid lipopolysaccharide antigen are prepared in TBS and each combined with an equal volume of 10% NHE. Each tube is then shaken thoroughly and incubated in a water bath for 2 hours at 37°C with occasional shaking. The resulting antigen-sensitized human erythrocytes (ASHE) are washed 3 times in TBS and resuspended to make a 1% suspension. Serial 2-fold dilutions (1:140-1:10240) of hyperimmune rabbit serum inactivated at 56°C for 30 minutes are prepared in TBS in sufficient quantity for testing each dilution of lipopolysaccharide antigen. For the titration, transfer 0.2 ml of each of the antiserum dilutions to Wasserman tubes to prepare a set of serum dilutions for each of the 5 ASHE preparations. Add 0.2 ml of each ASHE preparation to a row of serially diluted antiserum, mix and incubate in a 37°C water bath for 2 hours. Controls consist of (a) 1% NHE and serum, (b) 1% NHE and TBS, (c) ASHE and TBS. The HA titer is read as the reciprocal of the highest dilution giving complete agglutination of the red blood cells. A sample protocol showing standardization of *Shigella flexneri* 2a antigen is presented in Table I. In this instance the highest HA titer between 2560 and 10240 was given by antigen dilution 1:10 and this dilution would be the one used for all further tests with this particular lot of HA antigen.

Performance of the hemagglutination test. A preliminary HA test is carried out on all sera to be examined for presence of inhibitors, as antibodies would combine with any such material rendering detection of a circulating antigen by this method impossible. One volume of the optimum dilution of sensitizing antigen (see standardization of antigen) is mixed with an equal volume of 10% NHE and incubated in the water bath at 37°C for 2 hours with occasional shaking. The sensitized cells are washed 3 times with TBS and a 1% suspension prepared in TBS. Two-fold dilutions of the patient's inactivated serum (1:4-1:1024) are prepared in 0.2 ml amounts. A hyperimmune rabbit se-

TABLE II. HA Tests with Hyperimmune Rabbit Sera and 1% ASHE (Critical Dilutions To Be Used for HI Test).

E. coli	Final dilutions of hyperimmune rabbit serum								
serotypes	80	160	320	640	1280	2560	5120	10240	20180
01	+	+	C+	C+	C+	C+	C+	C±	C—
02	+	+	+	C+	C+	C+	C+	C+	C—
015	+	+	C+	C+	C+	C+	C+	C—	—

+, positive reaction; —, negative reaction; ±, incomplete agglutination, not considered positive reaction. C = Critical dilutions to be used in HI test.

Note: Lower dilutions may also be used if patient sera is sufficient.

rum control is also diluted as described in the section under antigen standardization. Two-tenths ml of ASHE is then added to each. Other controls are prepared as previously described. Tests are incubated for 2 hours at 37°C in a water bath and read.

Performance of HI test. Sera showing no hemagglutinins in the preliminary HA test may be tested for presence of inhibitors which are antigenically related to bacterial strains isolated from a patient's intestinal tract. Two-fold dilutions of hyperimmune rabbit sera are prepared of such strength that addition of an equal volume of patient's serum will yield critical dilutions. In this case, critical dilutions should include the end point, one 2-fold dilution beyond the end point, and 4 (or more) 2-fold dilutions preceding the end point as shown in Table II. These dilutions of hyperimmune sera are incubated together with a constant amount of patient's serum, undiluted or diluted 1:2 or more if amount of serum available is insufficient for the HI test. Similar dilutions of hyperimmune rabbit sera are incubated together with constant amounts of TBS. They are then placed for 1 hour in a 37°C water bath. ASHE are added and the tubes reincubated for 2 hours at 37°C and read. The hyperimmune rabbit sera are titrated each time the HI test is performed to determine exact titer levels. Controls are carried out as for the HA test. All patient sera yielding inhibition are retested to establish reproducibility of the results.

Discussion. There are several possibilities as to nature of the substance producing the inhibition. "Incomplete" antibodies against somatic (O) antigens of enteric pathogens have been shown by Neter *et al.*(11) to be present in human gamma globulin. The possible interference of such antibodies in the HA and HI tests was investigated as follows: (a) A modified Coombs test (antiglobulin hemagglutination) was performed with patient's sera in which presence of inhibitors had been established. Results did not indicate the presence of incomplete antibodies. (b) In the HI test, antihuman globulin was added to patients' sera prior to incubation with hyperimmune rabbit sera. No change in inhibitory activity could be demonstrated. In further efforts to study the inhibitor an attempt was made to sensitize NHE with the inhibitory substance present in the patients' sera. NHE and inhibitory sera were incubated together, the cells washed and hyperimmune rabbit sera against the bacteria isolated from the patients' fecal sample added. The negative result obtained might be interpreted as due to lack of sensitizing properties. On the other hand, the amount of inhibiting substance might have been insufficient to sensitize cells for the HA test even though it had such properties. In another experiment human antiglobulin was added to patient sera prior to an attempt to sensitize NHE with inhibitory substance. Otherwise the test was carried out as the previous one. Again negative results were obtained.

The possibility exists that blood group substances could be the interfering agents in our HA tests. "A" group substances are known to be widespread in nature. Springer *et al.*(12) have shown that enteropathogenic *Escherichia coli* 086 has a polysaccharide substance related to blood group B antigens. By use of human O Rh negative blood cells, however, it is hoped that cross reactions with these substances are eliminated.

As a further indication of the specificity of the reaction it should be pointed out that

HI tests for 2 or more bacterial strains isolated from an infant with diarrhea usually yield findings at considerable variance from one another. These would be difficult to explain except on the assumption that the HI test is specific. For example, the acute phase serum from a 5-months-old infant had no demonstrable antibodies or inhibitors for enteropathogenic *E. coli* 0127 isolated from his feces, but had a 3 tube inhibition against *E. coli* 015 which he also carried in his intestine. Similarly, the acute phase serum obtained from a 7-months-old child had a hemagglutinin titer of 128 for *E. coli* 018 and a 3 tube inhibition for *E. coli* 015 though both serotypes were isolated from his feces at the same time.

The HI test presented here has been successfully used to provide evidence for the presence in sera of patients with diarrhea of substances which are apparently related antigenically to bacteria present in their intestine. Results of such tests are presented elsewhere(13). The nature of the circulating inhibitor is unknown. It might be a complex substance or a simple sugar(14). Studies are continuing to further its characterization.

Summary. A simple hemagglutination-inhibition (HI) technic is presented which enables detection of inhibitors in patients' sera during diarrhea. These inhibitors thus far appear to be antigenically related to bacteria isolated from the patients' intestine at the same time and may well be substances derived from them.

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Infectious Agents in Infant Diarrhea: II. Serological Reactions with *Escherichia coli* 01 through 025. (26202)

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A number of *Escherichia coli* serotypes have been established as being associated with diarrhea in infants and young children, *i.e., E. coli* 0111:B_4, 086:B_7, 026:B_6, 0.55:B_5, etc.(1). Some of the *E. coli* serotypes 01 through 025 have also been described as possessing pathogenic properties(2,3). Grönroos(4), in an attempt to relate *E. coli* serotypes 01 through 025 to infant diarrhea, studied the feces of healthy and diarrheal infants and was unable to demonstrate significant differences in the two groups. Ørskov (5) carried out a similar study with 130 *E. coli* 0 immune sera and found that strains representing specific serotypes were found in about the same frequency in both groups of infants. The response of the host by antibody formation against "normal" *E. coli* serotypes 01 through 025 during attacks of diarrhea, however, has apparently not been determined. This study presents the results of an investigation of serological reactions of these serotypes both for antibody formation and for substances in the circulation which appear to be antigenically related to the *E. coli* serotypes isolated from the intestinal tract of infants.

Materials and methods. 1. Bacteriologic procedure. Rectal swabs were obtained from 118 infants and young children with diarrhea from Children's Hospital, Washington, D.C., and from 29 others from the Municipal Hospital, San Juan, Puerto Rico. These samples were frozen immediately in dry ice, then thawed within one week and cultured on eosin-methylene-blue, MacConkey and blood agar plates. All organisms were isolated, identified biochemically, and the serotypes determined using methods described by Edwards and Ewing(6). The first serum was drawn on admission during the acute place of the disease and a second serum, whenever possible, was obtained from 7 to 21 days later during convalescence.

2. Serologic procedures. a. Hemagglutination (HA) test: Neter *et al.*(7) have shown that the HA test affords a sensitive method of determining antibody response to *E. coli* infections. Our modification of this technic(8) was used for this study.

b. Hemagglutination-inhibition test: All sera negative for hemagglutinins at a final dilution of 1:8 were further tested for the presence of "inhibitors"(8,9) which were antigenically related to the homologous serotype of *E. coli* isolated from the infant's intestinal tract. Procedure for the HI test is published in the preceding paper of this series(8).

Results. Fifty-eight strains of *E. coli* 01 through 025 representing 17 O serotypes were isolated from the feces of 147 diarrheal infants and young children studied. Table I shows the number of strains studied and whether hemagglutinins and/or inhibitors were detected in the patients' sera. *E. coli* 015 was the most frequently encountered of the 17 serotypes found; 19% of the 58 *E. coli* strains were of this antigenic type. The serotypes not listed in Table I were isolated from only 1 or 2 patients. Of the total of 30 infants who had *E. coli* serotypes 01 through 025 present in their feces, and from whom acute and convalescent sera were available, 12 or 40% responded with a rise in antibody titer. Furthermore, of a total of 21 infants who had no hemagglutinins present in their acute phase sera, 14 or two-thirds had inhibitors present which were antigenically related to the *E. coli* serotype isolated from their fecal flora. Eleven convalescent sera were obtained from these same infants 1 to 3 weeks after the acute sera were drawn. Seven of the sera had inhibitors present, one showed a disappearance of inhibitor, and in 3 instances hemagglutinins had developed.

TABLE I. Distribution and Serologic Reactions of the Most Common O Groups of *E. coli* 01 through 025 in Infants with Diarrhea.

		No. of patients with	
E. coli serotype	Total No. of patients	HA antibody titer rise	Acute serum inhibitors
015	11	4/6*	5/5†
02	6	0/3	3/4
04	6	1/4	NS
06	5	0/2	2/2
07	5	1/4	1/3
017	4	1/3	1/3
08	3	1/2	NS
011	3	1/1	NS
016	3	1/2	1/2
019	3	2/3	1/2
Totals	49	12/30	14/21

* No. of patients with HA titer rise/No. of paired sera available for testing.

† No. of patients with serum inhibitors/No. of acute phase sera available for testing which contained no hemagglutinins.

NS = No serum available.

Note: Less common serotypes isolated: *E. coli* 013 and 025, 2 each; *E. coli* 01, 03, 012, 018 and 021, 1 each. (Only one of these strains, *E. coli* 012, had any significant serologic reaction, *i.e.*, inhibitors present in patient's serum.)

The serologic reactions with these *E. coli* groups can best be observed by examination of the results obtained with the most frequently encountered serotype, *E. coli* 015. These serologic findings are presented in Table II, together with the reactions of associated organisms and ages of the patients. Table II is arranged in sequence with those reactions listed first in which inhibitors were

TABLE II. Serologic Reactions with *E. coli* 015 and Associated Organisms.

Patient No.	Patient's age	*E. coli* 015 and associated organisms	Serologic findings: Acute serum titers HI*	Acute serum titers HA†	Convalescent serum titers HI*	Convalescent serum titers HA†
1	2 mo	015	4	0	4	0
		017	1	0	1	0
2	4 "	015	3	0	3	0
3	5 "	015	3	0	NS	NS
		0127	0	0	NS	NS
4	7 "	015	3	0	—	16
		018	—	128	—	128
5	8 "	015	3	0	—	32
6	10 "	015	—	256	NS	NS
7	1 yr	015	—	32	NS	NS
8	2 "	015	—	32	—	128
		Sh. flexneri 2	—	16	—	20480
9	3 "	015	—	32	—	128

* HI titer = No. of 2-fold dilutions of specific antiserum inhibited in HI reaction: Measurement of bacterial substance present in patient's serum.

† HA titer = Reciprocal of highest dilution yielding complete hemagglutination: Measurement of antibody.

NS = No serum available. — = Hemagglutinins present; test not performed.

present in both acute and convalescent phase sera; second are shown reactions in which inhibitors in acute phase sera were followed by antibody in the convalescent phase sera; and lastly are shown those reactions with antibodies present in acute phase sera which may or may not have shown an increase in the convalescent phase sera. When the data, arranged in sequence as in this table, are compared to the patients' ages, it can be seen that some association exists. Table II shows that in general the highest antibody titers were found in children over 7 months of age (Patients No. 4 through 9) and inhibitors were prevalent in the younger infants. Inhibitor persisted in the convalescent serum of one 8-months-old child harboring *E. coli* 06; otherwise it appeared only in the convalescent sera of infants 4 months of age or younger. On the other hand, hemagglutinin titer rises were not confined to older age groups, though they were much more frequently observed in children over 6 months of age as shown in Table III, which presents *E. coli* serotypes 01 through 025 which stimulated an antibody response and ages of the children from whom each was isolated. Patients No. 1, 2 and 3, 7 weeks old, 3 months old and 4 months old respectively, had *E. coli* serotypes 011, 016 and 08 isolated from their fecal samples during an attack of diarrhea and responded with a rise in hemagglutinating antibodies. Nine other children over 6 months of age experienced similar rises.

Discussion. An investigation of the serological reactions with *E. coli* 01 through 025 clearly revealed that a hemagglutinin titer rise for these organisms is a common finding when these serotypes are present in an infant's intestine during an attack of diarrhea. Utilizing the bacterial agglutination test Grönroos(4) was able to demonstrate ho-

TABLE III. *E. coli* Serotypes 01 through 025 Stimulating an Antibody Response and Ages of Patients from Whom Each Was Isolated.

Patient No.	Patient's Age	*E. coli* serotype	Hemagglutination titers: Acute sera	Convalescent sera
1	7 wk	011	8	128
2	3 mo	016	0	8
3	4 "	08	8	64
4	7 "	015	0	16
5	8 "	04	8	64
6	8 "	015	0	32
7	15½ "	019	0	16
8	20 "	019	32	256
9	22 "	07	128	1024
10	2 yr	015	32	128
11	3 "	015	32	128
12	7 "	017	16	256

0 = No hemagglutinins demonstrable in a serum dilution of 1:8.

mologous antibodies in the sera of only 7 of 115 patients positive for enteropathogenic *E. coli*. McNaught(10) found HA and agglutination tests to be of much the same sensitivity for detecting presence of serum antibodies to homologous strains of *E. coli*, but Neter *et al.*(11) in a study of the serological aspects of feeding experiments with human volunteers, found the hemagglutinin titers 5 to 20 times higher than O-agglutinin titers. In later studies with a variety of enteric pathogens Neter *et al.*(7) found hemagglutinin titer levels 2 to 64-fold higher in every case. Our experience with the HA test(9) corroborates that of Neter.

While many of the antibody elevations for *E. coli* 01 through 025 during diarrhea are of a low order, as revealed even by the sensitive HA test, many of the infants were quite young and some had no detectable antibodies prior to the diarrheal attack. The antibody rise is not due to a non-specific anamnestic reaction, as shown by Neter, *et al.*(7) who found no such rise to enteric pathogens other than the causative agent in cases of diarrhea, and also indicated in our results by the frequent antibody titer rises to only one of the enteric bacteria isolated from the intestine. A rise in antibody titer cannot be interpreted as proof that the corresponding organism was responsible for the disease state, but does clearly show that the *E. coli* serotype present in the infant's intestine, or some fraction thereof which is capable of stimulating antibody production, is passing the mucosal barrier. Such episodes provide one mechanism by which the child develops antibodies against so-called "normal" *E. coli* intestinal flora.

The second finding revealed by the serological investigation of *E. coli* 01 through 025 in infant diarrhea, would tend to corroborate the presumption that bacterial substances pass from the infants' intestine into the circulation. Substances were found to be circulating in the blood stream of the infant patient which were closely enough related to the organisms isolated from the intestinal contents to combine with specific antisera and inhibit the HA test. Such substances derived from bacteria might theoretically be any one of a number. Protein (12) and lipids(13) are capable of such inhibition by interfering with the sensitization of red blood cells by antigen. Since previously sensitized cells are added to the patient serum-rabbit antiserum in the HI test, lipids and proteins should not be capable of inhibiting the test through interference with red cell sensitization. However, material as simple as single sugars has been shown to neutralize agglutinins(14). Davies *et al.* (15) demonstrated that abequose inhibited HA tests performed with red blood cells sensitized with polysaccharide derived from *E. coli* 055 and 0111. The possibility that such simple sugars would be adsorbed in sufficient quantities to be inhibitory without being metabolized or eliminated, is remote. Bacterial polysaccharides, on the other hand, are known to be capable of inhibiting the HA test(16) and a relatively large amount circulating in the blood is known to temporarily depress the reticuloendothelial system thereby interfering with polysaccharide removal(17). Circulating polysaccharide has been detected in diseases such as pneumococcal pneumonia in sufficient quantity to give a positive precipitin test(18). Buxton (19) in experiments with *Salmonella gallinarum* in young turkeys found that, at the peak of infection, polysaccharide was present in the circulation in sufficient quantity to adsorb onto the turkeys' red blood cells. It might be speculated that in young infants the large numbers of *E. coli* found as high up as the duodenum(20) would provide optimum conditions for adsorption of inhibitory substances.

The adsorbed polysaccharide in the experiments of Buxton(19) was demonstrated by use of the antiglobulin hemagglutination test, *i.e.*, incomplete antibodies were demonstrated to have adsorbed onto the sensitized cells *in vivo*. Neter *et al.*(21) have shown such incomplete antibodies to be present in maternal and cord blood. It is to be noted that the most strongly inhibitory sera in the present series were from the youngest infants in whom incomplete antibodies would be expected to be highest. Theoretically such incomplete antibodies should be able to inhibit the homologous rabbit antiserum HA titer. However, experiments designed to further investigate this possibility(8) proved negative in a number of strongly inhibitory sera. Red blood cells from the acutely ill cases presented here were not obtained, so tests for sensitization of the cells *in vivo* could not be made. Preliminary studies with cases of colienteritis lead us to believe that it could occasionally occur(22).

The disease-producing mechanisms of established enteropathogenic *E. coli* serotypes are little understood. An antibody response to *E. coli* serotypes 01 through 025 during diarrheal attacks and presence in the circulation of substances related to these serotypes has been demonstrated. These findings may provide a clue to some of the dynamic interrelationships between infant host and organisms present in the intestine.

Summary. An investigation was undertaken to determine the immunologic sequence of events during attacks of diarrhea associated with the presence of "normal" fecal flora, *E. coli* serotype 01 through 025, in the intestinal tract. It revealed that 12 of 30 or almost half of those infants from whom acute and convalescent phase sera were available exhibited a rise in hemagglutinin titer. At some time during their illness a greater number, two-thirds, had inhibitors present in their sera which were antigenically related to the *E. coli* serotype isolated from their fecal flora.

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A New Method for Complete Hepatic Venous Occlusion in Dogs. (26203)

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Recent evidence has implicated obstruction of hepatic venous outflow as one of the important factors in pathogenesis of ascites in human cirrhotics(1). Previous experimental methods of producing ascites in dogs have utilized partial constriction of the supradiaphragmatic inferior vena cava, but with these methods, the effects of pure hepatic venous outflow cannot be assessed, since renal and adrenal venous hypertension are also produced. A 2-stage method has been devised for production of complete hepatic venous obstruction without these associated undesirable phenomena.

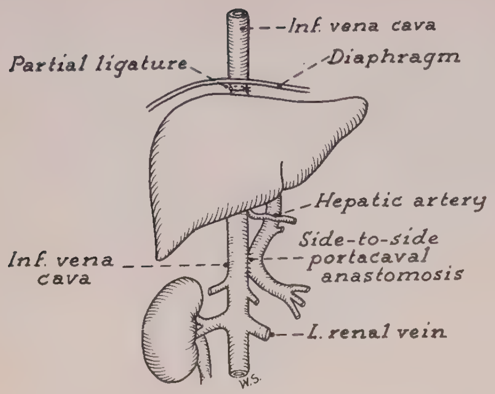
Methods. Healthy adult mongrel dogs were anesthetized with intravenous pentabarbital sodium. The first stage operation was carried out through a long right subcostal abdominal incision. A long side-to-side portacaval anastomosis was made and the inferior vena cava between liver and diaphragm was constricted to approximately ⅔ its diameter with a #0 silk ligature (Fig. 1).

Following a 2 to 3 week recovery period, the second stage operation was performed through a long thoracoabdominal incision utilizing very light anesthesia induced by a constant slow drip of a 2½% solution of sodium thiopental in isotonic saline. The inferior vena cava was transected immediately above the previous portacaval shunt. The hepatic side of the transected cava was doubly ligated and the distal end was anastomosed end-to-end with a crimped Dacron graft of suitable size. The supradiaphragmatic inferior vena cava was doubly ligated, transected, and the cardiac end anastomosed end-to-end with the upper end of the Dacron graft (Fig. 2). During this upper anastomosis, the descending aorta was occluded with tapes to prevent distal pooling. The graft was allowed to fill with blood by removing the vascular clamp on the abdominal vena cava, thus forcing any trapped air through the interstices of the graft before the upper vascular clamp was removed. The diaphragmatic incision was closed anteriorly only so that constriction of the graft was kept to a minimum.

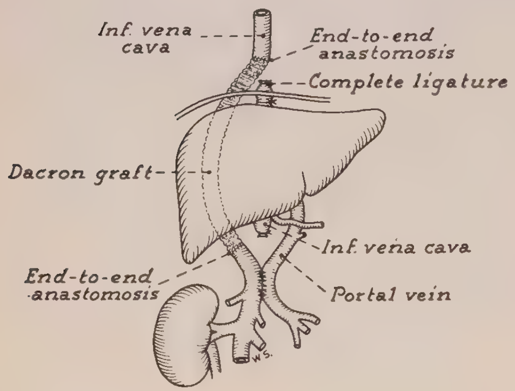
Liver biopsies were obtained at each of the 2 operations and at intervals after the second stage. Girth and weight were recorded preoperatively and at weekly intervals after operation. Total protein, serum albumin, serum globulin, thymol turbidity, and bromsulfalein excretion were measured preoperatively and at intervals postoperatively in surviving animals. Venograms were obtained *via* the femoral vein to determine vena caval and graft patency in second stage survivors.

Results. Two-stage hepatic venous occlusion was accomplished in 18 dogs. Ten survived 18, 27, 30, 31, 67, 84, 101, 113, 150, and 270 days after the second stage operation. One healthy survivor died at 270 days during an exploratory celiotomy. This animal had been in excellent health throughout, had patent graft and portacaval shunt, no ascites, normal liver function tests, and a normal liver biopsy. Two animals died 3 and 5 days after second stage operation of empyema. The remaining 6 dogs died of severe hypotension within the first 24 hours after the second operation.

Six of the 10 long-term survivors had patent Dacron grafts and portacaval shunts as demonstrated at autopsy and by pre-mortem venograms. Only one of these dogs developed ascites. This occurred 90 days after the second-stage operation and consisted of 5250 cc at autopsy on the 101st day. The 4 remaining long-term dogs developed ascites at varying intervals after operation, the onset coinciding with thrombosis of the Dacron graft as demonstrated by venography before and after the ascites appeared. The only consistent change in liver function was rapid decline of serum albumin after the development of ascites when it occurred. All of the 6



FIRST STAGE HEPATIC VEIN OCCLUSION ①



SECOND STAGE HEPATIC VEIN OCCLUSION ②

FIG. 1 and 2.

animals with patent grafts except the one which developed ascites had normal liver biopsies. The latter and the 4 dogs with thrombosed grafts showed marked sinusoidal congestion and lymphatic dilatation in their liver biopsies.

Discussion. The high immediate mortality rate caused by hypotension is unexplained, but is similar to the early deaths encountered by Child, *et al.*, when performing reversal of hepatic circulation(2). In all animals developing thrombosis of the Dacron grafts, massive progressive ascites developed, presumably because the portal vein could no longer function as an outflow tract for the liver(3). Only one of the 6 animals with patent Dacron vena cava grafts and portacaval shunts developed ascites. The reason for this one exception is unclear, particularly since the serum albumin remained relatively normal in this dog.

It should be recognized that although complete hepatic venous occlusion is accomplished anatomically by this operation, functionally, hepatic arterial blood is being shunted retrograde into the portal vein and thence into the vena cava through the portacaval shunt. The presence of the portacaval shunt is necessary since dogs do not survive acute hepatic venous occlusion without a shunt. The fact that most of the long term survivors did not develop ascites attests to the efficacy of such a shunt in decompression of the liver and may have clinical inferences supporting the concept that in patients with severe hepatic venous outflow obstruction manifested predominantly by ascites, side-to-side portacaval shunt rather than end-to-side shunt may be the procedure of choice. Since acute occlusion of the portacaval shunt in these dogs is uniformly fatal, further studies on the effects of gradual occlusion of the shunt or the portal vein cephalad to the shunt are being carried out.

Summary. 1. A 2-stage operation for production of pure complete hepatic venous outflow obstruction has been devised. Ten of 18 dogs survived from 18 to 270 days after operation. Crimped Dacron prostheses function satisfactorily in the low pressure venous system. 2. This preparation is presented as a satisfactory means of studying hemodynamic, hormonal, and chemical factors affecting development of ascites.

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Studies on Mechanism of Atabrine Inhibition of ATPase Activity of the Myofibrills.* (26204)

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It has been known since the early work of Wagner-Jauregg(1) that in the physiological pH range acridines precipitate ATP to form salts. Karremann, Mueller and Szent-Gyorgyi(2) found that acridine orange acting as a competitive inhibitor suppressed contraction of glycerol-extracted muscle fibre bundles. Sandow and Isaacson(3) explained the suppressive effect of acridine orange on muscle contraction on the basis of a photodynamic mechanism, and pointed out that some of the non-photodynamic effects of acridine orange on muscle are due to its Zn^{++} content. It therefore seemed to us that a study of the acridine inhibition of ATPase, as it exists in myofibrillar suspensions, would lead to information of greater relevance to the biochemical mechanisms involved. With a myofibrillar suspension, the problem of diffusion of both ATP and the acridine drug is removed. The preparation does not contain free Mg^{++} or Ca^{++} and the separate and combined participation of these cations can be conveniently studied. Atabrine was chosen for this study because it is obtainable as the hydrochloric acid salt which is free of Zn^{++} and because of its pharmacological importance.

Material and methods. Myofibrillar suspension (rabbit skeletal muscle) was prepared as previously described(4) except that 0.05 M borate buffer, pH 7.5, was substituted for the succinate buffer. ATPase determinations were carried out at room temperature (23-25°) in .05 M borate buffer, final volume 1.0 ml, pH 7.5, by measuring the amount of inorganic phosphate liberated in 5 mintues. The reaction was stopped by addition of 5% TCA solution. The deproteinized supernatant of the incubation mixture was treated with 0.1 ml charcoal slurry (1:2, v/v, activated charcoal and water) to absorb atabrine. ATPase activity is given in terms of μM inorganic phosphate liberated per mg of protein per minute (μM/mg/min). The composition of the individual incubation mixtures, in millimoles per liter, is specified below. Atabrine was kindly provided by the Dept. of Medical Research, Winthrop Laboratories, New York. Fluorometric measurements were made with a Coleman Electronic Photofluorometer, Model 12C using 14-212 and 12-221 filters.

Results. Curves A, B, and C of Fig. 1 show that in absence of Mg^{++} and Ca^{++} as activating cations, inhibition of ATPase activity by atabrine was demonstrated at low concentrations of ATP, but reversed at higher levels of ATP. On the other hand, in presence of 5 mM Ca^{++} (curve D, Fig. 1) atabrine at the concentrations indicated, now caused a 30% activation of ATPase (curve E, Fig. 1).

If 5.0 mM Mg^{++} was used instead of Ca^{++} as the activator of myofibrillar ATPase, atabrine behaved as a strong inhibitor. By increasing ATP concentration the inhibition caused by 1.0 and 1.5 mM of atabrine is reversed (curves B and C, Fig. 2). That caused by 2.0 mM or more of the drug is also reversed but to a slight extent (curves D, E and F, Fig. 2). Thus, in presence of Mg^{++}, atabrine behaved as a much stronger inhibitor than in its absence.

A comparison of curve A and B, Fig. 3, demonstrates stimulation of ATPase activity by Mg^{++} in presence of Ca^{++}, and under the conditions shown by curve A pronounced inhibition of ATPase activity could be demonstrated with atabrine as is illustrated in curves C, D, and E of Fig. 3.

In another experiment 300 mM KCl was added to each incubation mixture to insure an ionic strength above 0.40. ATP and Ca^{++} concentrations were fixed at 5.0 mM. Under these conditions 1.0 mM to 5.0 mM ata-

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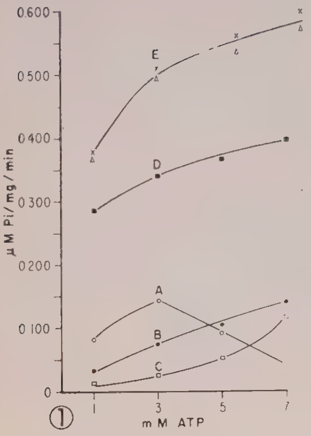
* Aided by grant from U.S.P.H.S.



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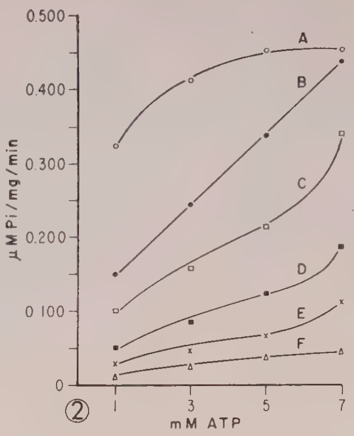

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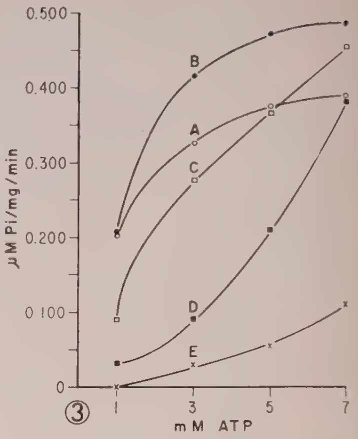

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FIG. 1. Curves A, B, C show effect of substrate concentration on atabrine inhibition of myofibrillar ATPase in absence of any added Mg^{++} or Ca^{++}. Each tube contained about 0.3 mg myofibrillar protein to which was added: Curve A (○) none, curve B (●) 2.5 mM atabrine, curve C (□) 5.0 mM atabrine. Curves C and D show effect of atabrine on 5 mM Ca^{++} activated myofibrillar ATPase at various substrate concentrations. Each tube contained about 0.3 mg myofibrillar protein and 5.0 mM Ca^{++} to which was added: Curve D (■) none, curve E (×) 2.5 mM atabrine, and (△) 5.0 mM atabrine.

FIG. 2. Inhibition of 5.0 mM Mg^{++} activated myofibrillar ATPase by atabrine at various ATP concentrations. Each tube contained about 0.3 mg myofibrillar protein and 5 mM Mg^{++} to which was added: Curve A (○) none, curve B (●) 1.0 mM atabrine, curve C (□) 1.5 mM atabrine, curve D (■) 2.0 mM atabrine, curve E (×) 2.5 mM atabrine, curve F (△) 5.0 mM atabrine.

FIG. 3. Effect of 0.5 mM Mg^{++} on 5 mM Ca^{++} activated myofibrillar ATPase at various ATP and or atabrine concentrations. Each tube contained about 0.3 mg myofibrillar protein and 5.0 mM Ca^{++} to which was added: Curve A (○) none, curve B (●) 0.5 mM Mg^{++}, curve C (□) 0.5 mM Mg^{++} and 1.0 mM atabrine, curve D (■) 0.5 mM Mg^{++} and 2.0 mM atabrine, curve E (×) 5.0 mM atabrine.

brine failed to produce any inhibition. If 0.01 mM to 0.5 mM Mg^{++} was added to incubation mixtures, 15-40% inhibition occurred, which remained unchanged when 2.5 mM atabrine was added together with the Mg^{++}. At high ionic strength atabrine, with or without Mg^{++}, was not inhibitory towards the Ca^{++} activated myofibrillar ATPase (myosin ATPase).

Karremann, Mueller and Szent-Gyorgyi (2) found that acridine orange can form a red fluorescent complex with ATP. They were able to show a similar red fluorescent complex in well-washed glycerol-extracted muscle fibres. Because of these results, they assumed that acridine orange and ATP might be bound by the same groups of the contractile proteins.

It was therefore thought desirable to investigate the influence of ATP, Ca^{++}, Mg^{++} and K^+ upon the fluorescence of the atabrine. Experiments were carried out in 1 x 10^{-5}M borate buffer, pH 7.5. The fluorescence of the atabrine is considerably increased by ATP and this increased fluorescence is quenched by Mg^{++}, Ca^{++} or K^+ (Table I). On the other hand, the same cations can quench the original fluorescence of the atabrine. These findings indicate formation of an atabrine-ATP complex which is destroyed by Mg^{++}, Ca^{++} or K^+ due to possible formation of a metal-ATP chelate complex and/

TABLE I. Influence of Presence of ATP, Ca^{++}, Mg^{++} and K^+, Either Alone or in Combination, on Fluorescence of Atabrine.

Atabrine	ATP	Ca^{++}	Mg^{++}	K^+	Relative fluorescence units
M/l					
1×10^{-5}	0	0	0	0	50
”	1×10^{-4}	0	0	0	90
”	”	5×10^{-5}	0	0	55
”	”	0	5×10^{-5}	0	52
”	”	0	0	1×10^{-4}	54
”	0	5×10^{-5}	0	0	22
”	0	0	5×10^{-5}	0	21
”	0	0	0	1×10^{-4}	22

or a metal-atabrine coordination complex.

Discussion. According to the results of ATPase determinations and fluorescence measurements, the inhibition of the myofibrillar ATPase at low ionic strength and in the absence of any added Mg^{++} or Ca^{++} might be explained satisfactorily by assuming complex formation between ATP and atabrine and/or between atabrine and the active center of the myofibrills. Mg^{++} and Ca^{++} are both known to be activators of myofibrillar ATPase at low ionic strength. They were equally effective in quenching the increased fluorescence of the ATP-atabrine complex, and so presumably reduce the stability of that complex. ATPase determinations have shown that atabrine was not inhibitory in the Ca^{++} activated system, but that its inhibitory action was potentiated if Mg^{++} was present. Klotz and Ming(5) found that Zn^{++} increased the binding of azo dyes to several proteins including enzymes. According to their interpretation, this effect can be adequately explained by coordination complex formation without assuming chelation. The experimental results described above led us to the assumption that a mechanism similar to that described by Klotz and Ming(5) is responsible for the increased myofibrillar ATPase inhibition in presence of atabrine plus Mg^{++}. (Fig. 2 and 3). It is interesting to point out the differences which exist between the mechanism of action of atabrine and several other known inhibitors of the myofibrillar ATPase system. According to the explanation offered by Perry and Grey (6), pyrophosphate and excessive amounts of ATP inhibit myofibrillar ATPase by binding Mg^{++} so that it is no longer available to the enzyme. The inhibitory action of ethylenediamine-tetraacetic acid is assumed to be a function of its great capacity to chelate Ca^{++} (7). The inhibition of these compounds was reversible if concentration of Mg^{++} and/or Ca^{++} was increased(6,7).

None of the above theories, except that of Klotz and Ming, are applicable to the phenomena observed in the study of the atabrine inhibition of myofibrillar ATPase. The chemical structure of atabrine makes it improbable that chelate formation with Ca^{++} or Mg^{++} can occur. Addition of a small amount of Mg^{++} (0.5 mM) in the 5.0 mM Ca activated system (Fig. 3), induced a similar degree of inhibition in presence of a given amount of atabrine as occurred in the 5.0 mM Mg^{++} activated system (Fig. 2) and in absence of Ca^{++}. This would indicate that under our experimental conditions 0.5 mM Mg^{++} was as equally effective as was 5.0 mM in producing a strong inhibitory compound with atabrine. This in turn indicates that the increased concentration of Mg^{++} or Ca^{++} did not reverse the inhibition of the atabrine plus Mg^{++}. This effect could be due to formation of a coordination complex between Mg^{++} and atabrine which is firmly bound to the myofibrillar ATPase, thus inhibiting the binding and splitting of the ATP. The negative results at high ionic strength are difficult to explain. By dissociating the actomyosin to actin and myosin, we deal with a different enzyme. The characteristics of the myosin ATPase concerning its ion activators, optimal pH etc., are quite different from that of the actomyosin(8). On the other hand, presence of the large amount of KCl (300 mM) greatly reduces the possibility of formation of an Mg atabrine coordination complex. Because of these considerations the lack of atabrine inhibition at high ionic strength can be explained in either of two ways, *i.e.,* the presence of a different enzyme or lack of formation of the Mg-atabrine coordination complex.

Summary. At low ionic strength, atabrine inhibition of myofibrillar ATPase activity was potentiated by Mg^{++}. Ca^{++} counteracted atabrine inhibition in absence but not in presence of Mg^{++}. The assumption of the occurrence of an Mg-atabrine coordination complex as the strong inhibitor is proposed as the best explanation of the experimental results. At high ionic strength, atabrine failed to inhibit Ca^{++} activated myofibrillar ATPase (myosin ATPase) with or without Mg^{++} being present.

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Ability of Isopregnenolone-21-Carboxylates to Block Renal Effects of Deoxycorticosterone and Aldosterone in Rats. (26205)

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Previous reports described the synthesis of a number of steroidal 17-spirolactones(1-3) with ability to block the acute renal electrolyte effects of aldosterone and aldosterone-like steroids in laboratory studies(4-7). The compounds studied clinically caused diuresis in edematous patients with associated hyperaldosteronism(8,9), demonstrating the therapeutic value of aldosterone-blocking agents (10). Our effort was recently directed toward synthesis of isopregnenolone-21-carboxylic acids, and the present report demonstrates that these compounds are potent deoxycorticosterone- and aldosterone-blocking agents in laboratory studies.

Materials and methods. The procedures for evaluating aldosterone-blocking activity have been described(5,6). Briefly, the method consisted of subcutaneously injecting groups of adrenalectomized rats (150 to 200 g) with isotonic saline and with an oil solution of 12 μg of deoxycorticosterone acetate (DCA). Test compounds were given additionally either as an injection or an oral dose in saline.* The animals were placed in metabolism cages after treatment for collection of a 4-hour sample of urine. No food or water was provided during collection period. In this assay, DCA alone reduces the urinary ratio of Na/K ions by causing simultaneously Na retention and K loss. Blocking activity was determined by reversal of the Na/K response and expressed in terms of proportion of the standard dose of DCA inhibited by the compound. Na and K analyses were done on a Beckman flame photometer.

Isopregnenolone-21-carboxylates were obtained by treatment of steroidal 17-spirolactones with a molar equivalent of methanolic Na or K hydroxide at reflux temperature for 30 minutes, and identified by elemental analysis and infrared spectral data. The following compounds were prepared for tests in animals: 3-oxo-17β-hydroxy-19-nor-17α-pregn-4-ene-21-carboxylic acid as the Na salt (I); 3-oxo-17β-hydroxy-17α-pregn-1,4-diene-21-carboxylic acid as the Na (IIa) and K (IIb) salts; and 3-oxo-9α-fluoro-11β, 17β-dihydroxy - 17α - pregn - 4 - ene - 21 - carboxylic acid as the K salt (III).

Results. Data demonstrating the DCA-blocking properties of the 4 compounds in adrenalectomized rats are shown in Table I, together with electrolyte data of untreated and DCA controls for reference. DCA alone reduced the Na/K ratio by inducing a strong retention of Na and some K loss. It can be noted that Compound I, IIb and III at the given doses blocked Na/K response primarily by changing Na output, with only slight and variable effects occurring on K excretion. At times, however, reversal of Na/K was obtained because of a greater effect on K than on Na excretion, as shown with a dose of 0.1 mg of Compound IIa. Over a wide range of effective doses, the maximal Na, K and Na/K responses achieved with the compounds approximated, but did not represent

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* Compounds are soluble in water to extent of about 15% by weight.

TABLE I. Action of Na and K Salts of Various Isopregnenolone-21-Carboxylic Acids in Blocking Renal Electrolyte Effects of DCA.

Treatment/animal		No. of animals	Mean log response		
DCA	Compound		Na	K	(Na × 10)/K
—	—	96	1.17	.83	1.34
12 μg	—	96	.77	.99	.75
12 "	.3 mg I	8	.91*	.95	.96*
12 "	.1 " IIa	8	.84	.81*	1.03*
12 "	.15 " IIb	8	.94*	.95	.99*
12 "	5.0 μg III	8	.95*	1.05	.91*

* $P < .05$ relative to DCA alone; P of .05 response = +.12 for Na, −.11 for K and +.11 for Na/K; Na and K = mM/l after dilution of sample/4 rats/4 hr to 50 ml; all treatment was by subcut. inj.

reversal beyond, those obtained in the untreated controls.

The dose for 50% block of the standard dose of DCA (median effective dose or MED) was determined by interpolation using cumulated dose-response data. Table II demonstrates that a dose of 0.33 mg of I was required by injection for 50% inhibition; orally, dosage for the same degree of blockade is believed to be > 2.4 mg. While IIa or IIb appeared to be more potent than I by a comparison of MED values, high variability precluded statistical significance for differences of the MEDS. The Na (IIa) and K (IIb) salts were approximately equipotent as blocking agents by injections or orally. Compound III, in the form of a K salt, gave the strongest activity in the series, producing 50% block at doses of 0.4 μg parenterally and 22 μg orally. Comparisons showed highly significant differences in MED values $(P<0.01)$ for III relative to I, IIa or IIb by either route of treatment. Absorption from the gastrointestinal tract appeared to be relatively incomplete for III, as indicated by MED ratios orally/parenterally of 55/1. IIa and IIb showed dosage ratios by the 2 routes of 5 to 7/1. There was no significant difference in slope of dose-response curves in the comparisons involving the compounds; thus, comments on relative potencies based on the MED values are expected to apply also at other levels than 50% block.

TABLE II. Relative DCA-Blocking Properties of Various Isopregnenolone-21-Carboxylates Given Subcutaneously and Orally.

	Median effective dose (MED)*	
Compound	Subcut.	Orally
I	.33 (.08 -1.30)	>2.4
IIa	.07 (.01 - .38)	.52 (.13 -2.05)
IIb	.15 (.07 - .32)	.82 (.37 -1.80)
III	.0004(.0001- .0020)	.022 (.005- .095)

* Dose in mg/rat for 50% block ($P = .02$ approx., using an 8-animal assay), or for reduction of the log Na/K effect of 12 μg of DCA to that obtained with 6 μg; values in parentheses describe 95% confidence interval.

TABLE III. Ability of An Isopregnenolone-21-Carboxylate (Compound III) to Block Renal Electrolyte Effects of Aldosterone.

Treatment/animal		Mean log response
Aldosterone	III	(Na × 10)/K
.3 μg	—	.85
.3 "	10 μg	1.04*
.3 "	100 "	1.02*

* $P < .05$ relative to aldosterone alone; least significant difference = +.11; 16, 12 and 12 animals for 0, 10 and 100 μg of III, respectively; all treatment was by subcut. inj.

Compound III was tested against *dl*-aldosterone-21-monoacetate† in similar adrenalectomized animals (Table III). Aldosterone alone gave a low urinary ratio of Na/K, by promoting Na retention and K loss. The effect on Na/K by aldosterone, however, was significantly reversed with III as a result of changes in both Na and K output; increasing the dose by 10-fold did not give a stronger blocking response. The isopregnenolone carboxylate was also given to similar animals in the absence of aldosterone or DCA (Table IV). It was ineffective on urinary Na, K

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† Kindly supplied by Dr. R. T. Hill, Nat. Inst. Health; the racemate showed approximately 40 times the activity of DCA as measured by Na/K response in adrenalectomized rats.

TABLE IV. Effects of An Isopregnenolone-21-Carboxylate (Compound III) Given Alone on Renal Electrolyte Excretion.

Treatment/animal	Mean log response Na	K	(Na × 10)/K
—	1.18	.81	1.37
10 μg III	1.30	.88	1.42
1000 " "	1.18	.75	1.43

Least significant change (P = .05) = ± .15 for Na, ± .14 for K and ± .13 for Na/K; 16, 12 and 12 animals for 0, 10 and 1000 μg of III, respectively; Na and K = mM/l after dilution of sample /4 rats/4 hr to 50 ml.

and Na/K over a wide range of doses.

Discussion. Chemically, the isopregnenolone-21-carboxylates may be considered as 17-spirolactones in which the lactone ring has been opened; the compounds may be expected to demonstrate aldosterone- and deoxycorticosterone-blocking properties. Our studies demonstrate that such an effect is present and that the compounds are pharmacologically equivalent to the 17-spirolactones. Available evidence indicates that the mechanism of electrolyte effects of isopregnenolone-21-carboxylates is by competition with aldosterone-like steroids for receptor sites in target tissues. Support for the concept is 2-fold: 1) compounds in the series block DCA and aldosterone without showing manifestations of physiological antagonism, and 2) basic structures of the compounds are close to those of steroidal spirolactones reported as competitive inhibitors(4-7). Other data also indicate that competitive interaction occurs in the kidneys, the generally accepted site of action of aldosterone-like steroids for effects on urinary electrolytes(11-14), and of steroidal spirolactones(15,16).

Compound III, the most potent in the series, is approximately 20 times more effective orally in laboratory tests than Aldactone[‡] ($P < 0.01$), a steroidal spirolactone(2,6) with diuretic properties in edematous states(10). However, it remains to be seen how these data will extrapolate to man.

Summary. Studies demonstrate that isopregnenolone-21-carboxylates are potent inhibitors of the renal electrolyte effects of deoxycorticosterone in adrenalectomized rats. Compound III, the most active in the series, was also effective against aldosterone. Available evidence indicates that the compounds affect electrolyte excretion by competition with aldosterone-like steroids at receptor sites in target tissue.

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[‡] Trademark of G. D. Searle and Co. for brand of spironolactone, or for 3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl)propanoic acid lactone.

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Domestic Fowl—Source of High Titer *P. tularensis* Serum for the Fluorescent Antibody Technic. (26206)

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Fluorescent antibody technic (FAT) has been offered as a rapid method of identification for many systems. Winter and associates (1) reported on identification of *Pasteurella pestis*. Recently, Smith, Marshall and Eveland(2) have reported on identification of *Listeria monocytogenes*. Most of these workers used hyperimmune rabbit serum prepared by multiple injection technics.

Thomason *et al.*(3) reported nonspecific cross-staining of *Escherichia, Salmonella, Shigella, Proteus* and *Vibrio comma* using heterologous sera. They referred to "natural antibodies" to certain strains of bacteria in rabbit serum. Other authors have reported the same or similar results(4,5). Finkelstein and LaBrec(6) obtained staining of various microorganisms with anti-cholera serum in smears of fecal suspensions from normal animals. In our laboratory, rabbit anti-*P. pestis* fluorescein conjugated globulin stains organisms found in normal pharyngeal washings. Rabbit and monkey anti-*Pasteurella tularensis* globulins (APTG) showed a high degree of nonspecificity at low dilutions and loss of specific staining at higher dilutions. The report of Abramoff and Wolfe(7) on the specificity and high titer of chicken serum to a single antigenic stimulus directed our attention to the domestic fowl in search for a high titered, more specific antiserum.

Materials and methods. Rabbit serum: Twenty-four hour glucose cysteine blood agar (GCBA) cultures of *Pasteurella tularensis* strain SCHU S-4 (PTS4)(8) were harvested in 0.5% formalin, held overnight at 5°C and diluted to a density of 9 x 10^8 organisms/ml (McFarland Nephelometer standard No. 3). Each animal was inoculated intravenously (IV) with 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 2.0 ml of this vaccine at 2-day intervals and exsanguinated one week following last injection. *Monkey serum*: Monkeys previously infected by exposure to an aerosol of PTS4 and fully recovered from the disease were bled and serum collected. *Avian serum*: Twenty-four hour GCBA cultures of PTS4 were harvested in saline and adjusted to 5 x 10^7 viable organisms/ml. Eight lb to 10 lb roosters of various breeds were each inoculated IV with 0.1 ml of this suspension, and after 9 days were exsanguinated and serum was collected. *Pseudomonas sp* (isolated from a patient) was cultured on heart infusion agar for 24 hr, harvested and triple washed in saline. A total dose of 10^{10} viable organisms was inoculated IV. Nine days post-inoculation the roosters were exsanguinated and serum collected. All sera were filtered through an EK Seitz pad and stored at –20°C in a mechanical freezer. Agglutination tests were performed to determine the titer of each serum.

Gamma globulin was precipitated by methanol fractionation according to Dubert *et al.*(9), maintaining a temperature of –5°C. The gamma globulin fraction was reconstituted to ⅝ the original volume with physiological saline adjusted with 0.1 N NaOH to pH 8.0. Gamma globulins were conjugated with fluorescein isothiocyanate according to the method of Coons and Kaplan(10) as modified by Riggs(11). Following overnight conjugation at 4°C the mixture was dialyzed seven days against normal saline.

Portions of each serum were adsorbed with whole mouse tissue powder according to Coons and Kaplan(10). Portions of avian and monkey PTS4 globulin were also adsorbed with *Pseudomonas sp* by adding 0.5 ml of packed, washed cells to each milliliter of globulin or globulin conjugate. The combination was placed in a shaker water bath at 37°C for 2 hours and followed by centrifugation at 24,000 G for 30 minutes in the cold (4°C) employing the International PR-2 centrifuge. The supernatant fluid was then

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TABLE I. Fluorescent Cross-Reactions Obtained with Anti-*P. tularensis* Globulin.

Test specimen*	Avian (1:5120†) Non-adsorbed 1:1	1:4	1:16	1:64	Avian *Pseudomonas*-adsorbed 1:1	1:4	1:16	1:64	Monkey (1:640) Non-adsorbed 1:1	1:4	1:16	1:64	Monkey *Pseudomonas*-adsorbed 1:4	1:8	1:16	1:32	Rabbit (1:1280) Non-adsorbed 1:1	1:8	1:16	1:32
P. tularensis	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	±	3+	2+	+	—	2+	+	±	—
Pseudomonas sp	2+	2+	+	+	—	—	—	—	2+	2+	2+	—	—	—	+	—	+	±	—	—
Neisseria sp	±	±	—	—	±	±	—	—	±	±	—	—	±	±	—	—	±	±	—	—
D. pneumoniae	±	±	—	—	±	±	—	—	±	±	—	—	±	±	—	—	±	—	—	—
Herellea sp	±	±	—	—	±	±	—	—	±	±	—	—	±	±	—	—	ND	ND	ND	ND
E. insidiosa	2+	+	+	—	2+	+	+	—	2+	+	+	—	+	+	+	—	ND	ND	ND	ND

* All other organisms examined were negative.
† Agglutination titer before fractionation.
ND = Not done.

filtered through an HA Millipore filter for clarification. Lissamine rhodamine counterstain was added to portions of each globulin according to Smith *et al.*(2).

Bacterial films were prepared from organisms grown on appropriate culture media. Twenty-four hour cultures were suspended in nutrient broth, normal saline and distilled water. A drop of suspension was placed on a slide and if not used immediately the films were stored at –25°C. Pharyngeal washes were collected from laboratory personnel in nutrient broth; organisms were added and mixing was accomplished with a syringe and 23-gauge needle. Smears were prepared with and without addition of PTS4, *Pseudomonas* and a mixture of the following organisms†: *Bacterium anitratum, Corynebacterium diphtheria, Diplococcus pneumoniae*—Types III, VI, XIV, XXIII, *Erysipelothrix insidiosa, Herellea sp, Listeria monocytogenes, Mimae sp, Neisseria perflava, Salmonella paratyphi, Shigella dysenteriae, Shigella flexneri, Staphylococcus pyogenes, Streptococcus pyogenes.*

All films to be examined were fixed in 10% formalin (ACS) (pH adjusted to 7.2-7.4 with 0.1 N NaOH). Period of fixation was 10 minutes, followed by a 10-minute normal saline wash. While still moist, 2 to 3 drops of globulin conjugate (pH 7.2) were spread over the surface of the film. The preparation was placed in a moist chamber and incubated at 37°C for 30 minutes. The slides were then rinsed to remove excess conjugated globulin, placed in flowing normal saline solution for 15 minutes and mounted in phosphate buffered saline-glycerin (pH 7.4-7.6).

A Zeiss fluorescent microscope (Model GF) equipped with an OSRAM HBO 200 lamp was used. Our criteria for positivity required that the particle specifically fluoresce with the Schott filter combinations BG12-GG4 as well as with the BG12-OG4-GG4.

Results. Fluorescent antibody cross-reac-

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† Obtained from Division of Immunology, Walter Reed Army Inst. of Research, and Bacteriology, Immunology, and Infectious Disease Branch, Armed Forces Inst. of Pathology, Washington, D. C.

TABLE II. Cross Agglutination Titers of *P. tularensis* and *Pseudomonas sp.*

	Agglutinin titers Organisms	
Avian serum	*P. tularensis*	*Pseudomonas sp*
P. tularensis		
Pre-immune	<1:10	<1:10
Immune	5120	320
Pseudomonas sp		
Pre-immune	<1:10	<1:10
Immune	320	5120

tions of APTG with *Pseudomonas, Neisseria* and *D. pneumoniae* organisms (Table I) occurred with monkey, rabbit and avian globulin. Use of higher dilutions in an effort to eliminate or reduce these cross-reactions was successful only with the avian serum. Normal and pre-immunization serums of all 3 species gave a weak (±) fluorescence with *Neisseria* and *Diplococcus*.

The fractionated and conjugated avian APTG was examined to determine its maximum working dilution with *P. tularensis* and *Pseudomonas*. At a dilution of 1:512 in phosphate buffered saline (pH 7.2) a 3+ reaction to *P. tularensis* and a ± reaction to *Pseudomonas* were obtained. The cross-reaction with *Pseudomonas* was eliminated by specific adsorption with washed, homologous living cells. The working dilution for *P. tularensis* following this adsorption was reduced to 1:128.

Using 1:1, 1:2 and 1:4 dilutions of unadsorbed APTG, cross-reactions occurred with *P. pestis* (non-encapsulated organisms only), *Pseudomonas, Neisseria* and *Diplococcus*. At a 1:64 and 1:128 dilution only the *Pseudomonas* cross-staining persisted. Cross-reactions with other organisms listed above using *Pseudomonas*-adsorbed globulin occurred only with *E. insidiosa* and *Herellea sp* (Table I). These cross-reactions were eliminated at a 1:64 dilution of globulin. Bentonite (12) treatment, mouse tissue powder adsorption and addition of lissamine rhodamine counterstain to non-*Pseudomonas*-adsorbed globulin were studied as a means of reducing cross-reactions, but showed no advantage over dilution of *Pseudomonas*-adsorbed globulin and did not eliminate the *Pseudomonas* or *E. insidiosa* cross-reactions.

Using *Pseudomonas* and *P. tularensis* agglutinating antigens and *P. tularensis* and *Pseudomonas* antisera in agglutination tests, specific cross-reactions were noted (Table II). Further, PTS4 organisms were stained with conjugated avian *Pseudomonas* globulin.

To examine the possible role of diluents, *P. tularensis* and *Pseudomonas* organisms suspended in nutrient broth, distilled water and physiological saline were stained with avian APTG. No difference in intensity of staining and clearness of background was detected.

Slides prepared from normal pharyngeal washes with and without addition of PTS4 and a mixture of other organisms were stained with normal, heterologous immune and *Pseudomonas* adsorbed avian anti-*P. tularensis* globulins. Lissamine rhodamine was added to all APTG. Of 60 coded slides 15 of 16 positive and 43 of 44 negative slides were read correctly.

Discussion. When examining pure cultures, cross-reactions present little or no problem; however, with the FAT for rapid identification, any specific fluorescence may result in a false positive diagnosis unless the particle is morphologically distinguishable or the cross-fluorescence can be eliminated. Past experience in this laboratory with monkey and rabbit serum precluded identification of *P. tularensis* and *P. pestis* in simulated and actual clinical specimens because of our inability to prepare suitable specific antisera. Specific immune monkey and rabbit sera, although possessing high agglutination titers, did not allow sufficiently high fluorescent antibody working dilutions to permit the necessary manipulations. Normal rabbit and normal monkey globulins exhibited fluorescent reactions with a variety of organisms commonly found in clinical specimens.

Abramoff and Wolfe(7) report on the high titered, specific response of the domestic fowl to a single antigenic stimulus, "Although multiple injections are usually effective in enhancing antibody titers . . . such procedures tend to reduce the specificity of antisera." Therefore roosters were given a single IV dose of living *P. tularensis* organisms and an excellent agglutinating response was obtained. This avian globulin conjugate exhibited specific fluorescent antibody staining of *P. tularensis* at a dilution of 1:512.

Although a 4+ staining of *P. tularensis* occurred at a 1:512 dilution of avian globulin, a weak (±) reaction with *Pseudomonas* persisted. At lower dilutions, *Neisseria* and *Diplococcus* exhibited specific fluorescence to APTG of all 3 species but the avian globulin had a sufficiently high, 4+, working dilution to eliminate these cross-reactions. To eliminate the weak cross-reaction of *Pseudomonas,* specific adsorption with living *Pseudomonas* organisms was essential. This adsorption lowered the dilution at which 4+ staining of *P. tularensis* occurred to 1:128 and eliminated the *Pseudomonas* cross-reaction. This suggested a possible common antigen between *P. tularensis* and this strain of *Pseudomonas.* Comparative agglutination studies supported this hypothesis (Table II). Fractionated and conjugated avian *Pseudomonas* globulin also gave specific fluorescence with *P. tularensis* organisms. Specific adsorption with living PTS4 organisms eliminated this cross-staining.

In addition to the *P. tularensis* and *Pseudomonas* antiserums reported above, studies are now in progress using rooster serums prepared against: *P. pestis, Bacillus anthracis,* Rift Valley fever virus, Venezuelan equine encephalomyelitis virus, *Coccidioides immitis* and human, burro, monkey, mouse, rabbit and guinea pig globulins.

Summary. A method for producing a high titer, specific anti-*P. tularensis* globulin is presented. Roosters immunized with living *P. tularensis* organisms produced an antiserum that is more specific and has a higher working dilution than rabbit and monkey sera. The suspending medium for the organisms does not affect degree and intensity of specific staining or working dilution of serum. Cross-reactions obtained suggest that a specific antigenic relationship exists between *P. tularensis* and the *Pseudomonas* used in this study. This cross-reaction was eliminated by specific adsorption with heterologous living organisms.

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A Simple Fractionation Method for Preparation of Fluorescein-labeled Gamma Globulin.[*] (26207)

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To reduce the amount of non-specific staining in the fluorescent antibody technic, labeled gamma globulin has been used instead of whole serum. However, most methods for preparation of labeled gamma globulins in addition to being time-consuming, result in a loss in some of the antibody active fractions. The observations of Levy and Sober(1) using an anion exchange adsorbent, diethylamino-ethyl cellulose (DEAE) to prepare relatively pure gamma globulin, suggested that this procedure could be extended to preparation of fluorescein-labeled gamma globulins, since the high capacity of the cellulose ion-exchange adsorbent, together with the relatively mild conditions of salt and pH required for adsorption and elution resulted in almost quantitative recovery of purified antibody. The procedure to be described, therefore, represents a modified separation procedure employing the cellulose ion exchanger DEAE to recover labeled gamma globulin from previously labeled whole serum.

Materials and methods. Four different hyper-immune sera were conjugated with fluorescein isothiocyanate (FITC) and fractionated through DEAE columns as described below. These sera consisted of rabbit anti-*Hemophilus pertussis,* rabbit anti-*Salmonella typhosa,* monkey anti-polio virus type 3, and ferret anti-Asian influenza virus.

Conjugation procedure. Serum samples were conjugated in the usual manner(2,3) and were then dialyzed overnight against 0.0175 M PO_4 buffer, pH 6.3 in the cold. As a control, a sample of each serum was treated in the same manner, but with omission of the fluorescein isothiocyanate dye.

Ion exchange adsorbent. The anion cellulose exchanger, DEAE (40-100 mesh, 0.96 meq/g), was prepared by the method of Levy and Sober(1). After equilibration with the starting buffer, (0.0175 M phosphate buffer, pH 6.3), the exchanger was packed into glass columns under mild air pressure. For every 2 ml of undiluted conjugated serum a column of about 1 cm in diameter and 5 cm in height was prepared.

Fractionation procedure. A stepwise elution schedule with eluting solutions consisting of increasing concentrations of NaCl in 0.0175 M phosphate buffer, pH 6.3, was employed to fractionate both labeled and non-labeled sera. The fractionation process was carried out at 4°C with diluted serum, using relatively rapid flow rates of from 1 to 2 ml per minute. To each adsorbed column 8 ml of eluting fluid was added and the effluent collected as four 2 ml fractions, and their relative protein content determined by measurement of ultraviolet absorption at 280 mμ in the Beckman DU spectrophotometer.

Antibody assay and staining efficiency. A representative effluent fraction of each different eluent concentration was dialyzed against 0.01 M phosphate buffered saline, pH 7.0, and its antibody content and staining ability were determined. Antibody content of the fractions of the 2 anti-bacterial sera was determined by the tube agglutination technic, anti-influenza virus serum by the hemagglutination-inhibition procedure, and anti-poliovirus serum by the tube neutralization technic using monkey kidney cells. To determine the staining efficiency of the fractions obtained, smears of the bacterial antigens were made, heat fixed, and stained. Anti-poliovirus serum fractions were tested by scraping monkey kidney cells infected with type 3 poliovirus from the walls of the tube culture, suspending them in phosphate buffered sa-

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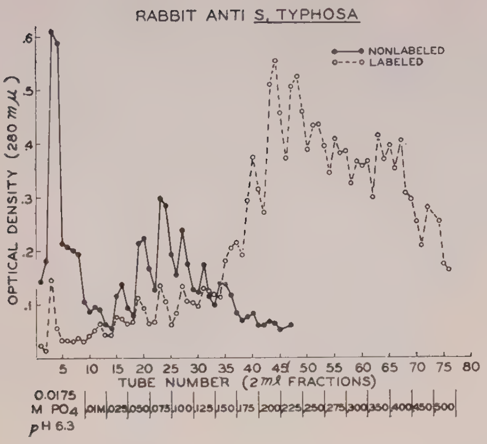
[†] Post-doctoral Trainee under U. S. Public Health Training Grant.



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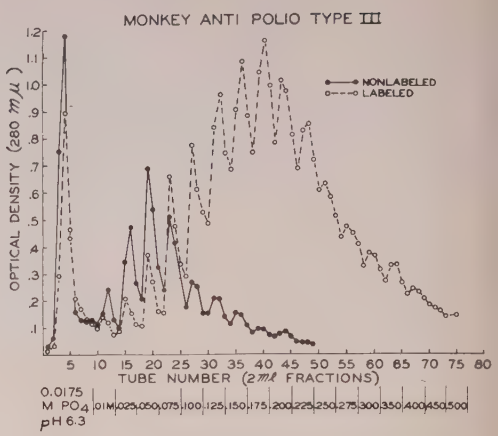

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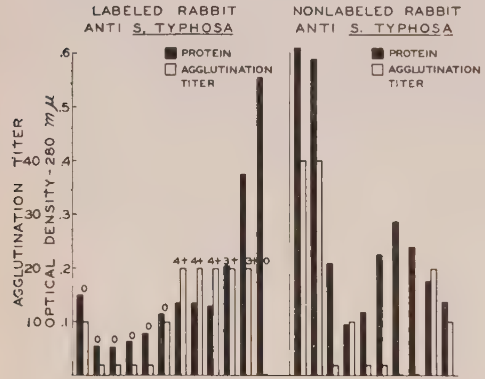

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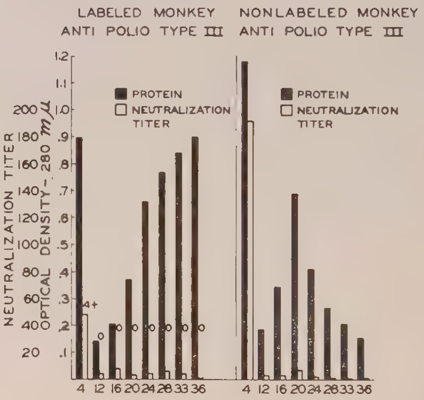

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FIG. 1. Rapid stepwise anion exchange chromatography of fluorescein isothiocyanate-labeled and non-labeled rabbit anti-*S. typhosa* sera. Two ml of conjugated serum applied to a 1 × 5 cm column of DEAE cellulose. Fractions (4 × 2 ml) collected at a rate of 2 ml/min. Eluting buffer was 0.0175 M Na-phosphate, pH 6.3, with stepwise changes in NaCl concentration and constant pH. Temp. 4°C.

FIG. 2. Rapid stepwise anion exchange chromatography of fluorescein isothiocyanate-labeled and non-labeled monkey anti-poliovirus type 3 sera. Two ml of conjugated serum applied to a 1 × 5 cm column of DEAE cellulose. Fractions (4 × 2 ml) collected at a rate of 2 ml/min. Eluting buffer was 0.0175 M Na-phosphate, pH 6.3, with stepwise changes in NaCl concentration and constant pH. Temp. 4°C.

FIG. 3. Antibody activity in relation to staining efficiency and protein content of serum fractions obtained by anion exchange chromatography. The tube numbers correspond to those found in Fig. 1. Staining efficiency: 4+ excellent, 3+ good, 2+ fair, 1+ poor, and 0 none.

FIG. 4. Antibody activity in relation to staining efficiency and protein content of serum fractions obtained by anion exchange chromatography. The tube numbers correspond to those found in Fig. 2. Staining efficiency: 4+ excellent, 3+ good, 2+ fair, 1+ poor, and 0 none.

line, pH 7.0, and making smears of the suspended cells. After air drying, the smears were fixed in acetone for 10 minutes, air dried, and stained. The staining efficiency of anti-influenza virus serum fractions was carried out by Dr. A. Inglot of this laboratory by a method yet to be published. For both bacterial and viral antigens, the staining procedure was that essentially described by Riggs *et al.*(2).

Results. Typical fractionation patterns obtained by this method are shown in Fig. 1 and 2. Two definite peaks are obtained with non-labeled sera, which on further separation with increasing concentrations of salt yielded no additional peaks. However, after labeling with FITC, additional protein peaks are obtained within the same range of salt concentrations (0.01 M-0.5 M NaCl). Whereas all antibody activity of the non-labeled sera appeared within the effluents obtained with the original phosphate buffer, that of the FITC-labeled rabbit sera tends to be more widely distributed (Fig. 3,4). This spread was not observed with monkey serum. Essentially all antibody activity of the labeled rabbit sera can be recovered with higher concentrations of salt (up to 0.175 M NaCl-Tube 40); however, as will be described below, staining with the antibody-containing higher salt concentration fractions (0.15 M NaCl), results in an increased amount of non-specific staining. When representative antibody-containing fractions of the 2 peaks obtained from non-labeled sera were examined by paper electrophoresis, it was found that they belonged to the gamma globulin component of serum. Fractions from labeled serum obtained at molarities corresponding to these peaks also showed electrophoretic mobility of gamma globulin. However, because of the labeling process the mobility is somewhat slower than with native serum.

The staining efficiency of the various antibody-containing fractions obtained was graded as follows: 4+ excellent, 3+ good, 2+ fair, 1+ poor, and 0 none. With monkey anti-poliovirus and ferret anti-influenza virus sera, only the fractions eluted with the starting buffer (0.0175 M phosphate buffer, pH 6.3) exhibited staining ability, while the rabbit anti-bacterial sera exhibited staining ability only with fractions eluted by higher salt concentrations, the strongest staining being observed in the 0.075 to 0.125 M NaCl fractions. With fractions above 0.15 M NaCl, a consistent increase in amount of non-specific staining was observed. Staining of the homologous antigens was well defined in that the background fluorescence indicating presence of unbound dye or dye bound to non-antibody proteins was not observed. Staining of impression smears made from *H. pertussis*-infected mouse brains, reveals bright specific staining of the bacterial antigen with little or no fluorescence in non-infected cells or other background material (Fig. 5). With the anti-poliovirus serum fractions, background fluorescence also was reduced, and positively stained cells could easily be distinguished from negative ones in the same microscopic field.

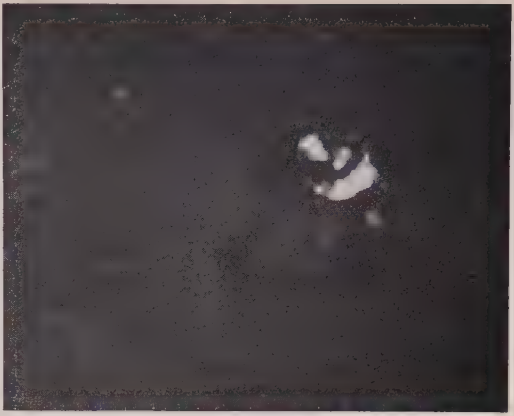

FIG. 5. Impression smear of mouse brain infected with *H. pertussis* and stained with labeled gamma globulin (0.125 M NaCl eluate) fractionated through the DEAE cellulose column.

As a routine procedure for preparation of labeled gamma globulin, it is now suggested that immune serum after appropriate dilution with physiological saline and conjugation with FITC, be dialyzed overnight at 4°C, against 0.125 M NaCl in 0.0175 M phosphate buffer, pH 6.3. After dialysis the conjugate is put through the DEAE-cellulose column. For every 2 ml of undiluted starting serum, a column of about 1 cm diameter and 5 cm in height is prepared. Size of the column can be adjusted to amount of starting serum available. The non-absorbed gamma globulin fractions are collected and the column washed through with more of the eluent (0.125 M NaCl in 0.0175 M phosphate buffer, pH 6.3). After determination of protein and FITC content of each aliquot by their absorption at 280 mμ and 490 mμ respectively, aliquots showing the highest protein and dye content are combined, and dialyzed overnight at 4°C against phosphate buffered saline, pH 7.0. Concentration of the fractions can be carried out by pervaporation, ultra-filtration, or dialysis against PVP (polyvinylpyrrolidone), although if a serum of high titer is used initially, the dilution factor may be ignored.

By using whole labeled immune serum, advantage is taken of the fact that the gamma globulins are isolated at the same time as unbound dye is removed from the conjugate. The alternative of first fractionating the serum and then labeling the gamma globulins has also been carried out although the extended periods of dialysis to remove unbound dye from the conjugate are not curtailed by this procedure. One may, however, refractionate on the DEAE column after labeling, thereby further purify the antibody and remove unbound dye.

The procedure described can be used readily in any laboratory, and presents several advantages over the usual chemical or physical methods for isolation of labeled antibody. Aside from the relative ease and expediency of the method, mildness of the conditions thus required for isolation of FITC-labeled gamma globulin with specific antibody activity, should be appreciated. Also important is the marked reduction of non-specific fluorescence by elimination of non-antibody containing serum components. The additional steps of absorption with tissue powders necessary in other procedures to reduce non-specific staining could often be eliminated.

Discussion. The change in fractionation behavior of serum onto DEAE cellulose column after labeling with FITC is of considerable interest. Our investigation has once again reaffirmed the heterogeneity of gamma globulin(5). Only 2 components, both electrophoretically identified as gamma globulin, are obtained by elution under these conditions; the first component contains the majority of the specific antibody. Labeling of the serum with FITC, however, brought about a change in both the fractionation behavior and physiological activity of the gamma globulin. In addition to a depression in protein content of both the early peaks noted in native serum, further fractionation with increasing salt concentrations revealed that additional serum components could be eluted. Preliminary investigations suggest that these additional proteins are composed of α and β globulins and albumin. An explanation for this change in chromatographic behavior is being pursued. With regard to antibody behavior it is of interest that the shift in antibody character requiring a higher concentration of salt for elution is found only with labeled rabbit sera. Antibody activity in ferret anti-influenza virus and monkey anti-poliovirus sera was essentially unaltered. Whether this difference in labeled antibody behavior is due to a species difference in sera, or to the different antigens used in the immunization process is yet to be determined. However, the heterogeneity in antibody activity of immune sera against different antigens when subjected to fractionation on cellulose columns has also been described by Fahey and Horbett(4) and Sober and Peterson(5).

Summary. A relatively simple method for separation of fluorescein isothiocyanate-labeled gamma globulin from serum has been presented. The procedure has been applied to a number of labeled anti-bacterial and anti-viral sera, and the labeled gamma globulin obtained gave definite specific fiuorescence when used in the staining procedure with markedly low background fluorescence.

The authors wish to acknowledge the technical assistance of Mrs. F. Malone.

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Modification of Rabbit Aortic Strip Technic for Catecholamine (4-point) Assay and Pharmacological Studies.*† (26208)

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In recent years catecholamine studies have been frequently performed on the isolated rabbit aortic strip, which gives a graded contractile response. It has been pointed out repeatedly that the rabbit aortic strip might serve as a reliable preparation for quantitative determinations(1-4). However, the waiting periods both for relaxation before starting drug applications and between a series of doses are many hours; in addition, this organ loses sensitivity after contraction by a supramaximal chemical stimulus. These factors limit seriously both the number of doses which can be applied to any one strip as well as the constancy of successive responses. The fully relaxed state, though, can quickly be reached by introduction of a small change in technic and consistent responses obtained by application of a dose at intervals as short as 7 minutes. With this modification, the rabbit aortic strip becomes suitable for a variety of uses.

Methods. A strip of rabbit aorta is set up as described by Furchgott and Bhadrakom (1). From a segment of thoracic aorta cut spirally, 4 pieces, 3 mm wide and 3 cm long, are obtained. Preparations, fresh and refrigerated for 24 hours, respond equally well. A strip is suspended in a 30 ml organ bath at 37°C in Krebs bicarbonate Ringer solution of pH 7.4 and aerated with 95%O_2-5% CO_2. The contractions are recorded on a smoked-paper kymograph with a light frontal writing lever at an amplification ratio of 1:20. The tissue is stretched with 2 to 2.5 g weight. For initial relaxation, the aortic strip is repeatedly, gently stretched by pulling down the writing lever. The organ is permitted to adjust to its resting length by tapping the bar to which the lever is attached, and the kymograph is started to mark a segment of baseline. While the drum is stopped, a dose of drug is added and the organ permitted to contract for 3 minutes during continuous tapping. The drum is started again to mark the 3-minute contraction height by a short horizontal line. The tissue is washed with drug-free Ringer solution, and 1 minute later gently stretched several times. The strip returns consistently to its resting baseline. Fresh, aqueous solutions of sympathomimetic amine salts are made up daily and stored at 0°C during use.

Results. 1. Assays of catecholamines. a) A graded dose-response relationship can be obtained easily. In a typical experiment, 5 doses, each twice the amount of the previous one, of l-epinephrine bitartrate 0.2 μg/30 ml bath (0.007 μg/ml), 0.4, 0.8, 1.6 and 3.2 μg cover the range from minimum to maximum response. Such doses, applied regularly at 7-minute intervals, can be repeated on the same strip throughout the day. b) Assay by alternating doses. A dose-response relationship as described under a) is obtained separately with a standard l-epinephrine solution and with the unknown solution to be tested. A dose of the known solution is chosen which will give about 50% of maximum contraction and is alternated with slightly more and slightly less effective doses of the unknown until a close match is obtained. c) 4-point log dose-response, parallel line assay. Having obtained dose-response series of the standard l-norepinephrine bitartrate monohydrate and unknown solutions, as described under b), a low (S_L) and a high dose (S_H) of the standard solution were selected to induce contractions to approximately 30 and 70%, respectively, of the maximum response. Similarly active low

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TABLE I. Aortic Strip Responses of First Assay.

Dose:	.80 μg (S_L)	3.2 μg (S_H)	1.1 ml (U_L)	4.4 ml (U_H)
Replications		Responses (mm)		
1	9.0	28.0	10.0	25.0
2	11.5	30.0	8.0	26.0
3	7.0	31.0	8.0	26.0
4	11.0	30.0	9.0	24.0

(U_L) and high (U_H) doses of the unknown solution were selected, so that $\frac{S_H}{S_L} = \frac{U_H}{U_L}$.

In the first assay, 4 replications of each of the dose levels, S_L, S_H, U_L, and U_H, were applied to the aortic strip. The order of application of the doses was essentially random. Dose levels and responses of this first assay are given in Table I.

The potency estimate of the unknown, in terms of the standard, is 59.3%. The 95% fiducial limits for the potency are 52.9 and 66.4%, calculated as described by Finney (5). A statistical analysis of variance of the data shows a significant departure from parallelism of the 2 dose-response lines ($p < 0.05$). This non-parallelism is probably due to extension of the high dose of the unknown solution (U_H) beyond the linear portion of the log dose-response curve. Estimate of potency and fiducial limits for this assay should therefore be considered as close approximations rather than as exact estimates.

A second assay was run with a ratio of high to low dose of 3 to 1, rather than 4 to 1, the ratio of the first assay, to increase the likelihood of obtaining responses within the linear portion of the log dose-response curve. The 4 doses were again replicated 4 times, but in this assay a latin-square design was used to predetermine the order in which the doses were applied to the aortic strip. Any effect on responses due to the order in which each dose was applied would, therefore, be cancelled out. It also became possible to measure an order effect on responses, should it exist. Responses to each of the 4 doses and the latin-square design used in the second assay are shown in Table II.

A statistical analysis of variance of the data shown in Table II, given in Table III, confirms the existence of a real log dose-response parallel line relationship. It demonstrates that each replication gives essentially similar average aortic strip responses and that the order of doses does not show a demonstrable influence on these responses.

TABLE III. Analysis of Variance of Data of Table II.

Source of variation	Degrees of freedom	Mean square	F-ratio
Total variation	15	—	—
Regression	1	1164.14	Sig. at $p < 0.001$
Drugs	1	92.64	" " $p < 0.01$
Parallelism	1	11.68	Not sig.
Replications	3	3.52	" "
Order of doses	3	1.85	" "
Error	6	6.23	—

Fiducial limits were calculated by combining the last 3 sources of variability for the experimental error. Estimate of potency from the second assay is 49.5%, and 95% fiducial limits are 31.4 and 77.4%. d) Assay of l-norepinephrine added to normal urine. l-Norepinephrine bitartrate monohydrate from an aqueous solution as well as from a solution in normal urine is adsorbed on alumina(6,7). It is then eluted and the 2 eluates, tested on the rabbit aortic strip, induce contractions closely agreeing with each other. Normal urine without added norepinephrine is processed and applied in the identical manner; this control solution causes no significant contractions. It is therefore suggested that the rabbit aortic

TABLE II. Aortic Strip Responses of Second Assay.

	Order of administration of doses and responses							
Replications	1	mm	2	mm	3	mm	4	mm
1	S_L	12.0	S_H	31.0	U_L	10.0	U_H	22.0
2	S_H	32.0	U_H	20.0	S_L	10.0	U_L	9.0
3	U_L	8.0	S_L	12.0	U_H	28.0	S_H	29.0
4	U_H	26.0	U_L	7.5	S_H	30.0	S_L	13.0

$S_L = .90\ \mu g$ $S_H = 2.70\ \mu g$ $U_L = 1.33$ ml $U_H = 4.00$ ml strip could be used for assaying catecholamines in urine.

2. Pharmacodynamics of sympathomimetic amines can also be conveniently studied.

Dichloronorepinephrine hydrochloride (DCN) (50, 100 and 200 μg/30 ml bath) potentiates contractile responses to norepinephrine. These doses of DCN by themselves do not contract the strip significantly. However, in their presence, norepinephrine (0.027 μg/ml) gives much higher contractions. Control doses of norepinephrine in absence of DCN are given alternately. With increasing doses of DCN, increasing potentiation is observed.

3. Inactivation of sympathomimetic amines can also be demonstrated on the aortic strip. Ten ml of Krebs bicarbonate Ringer solution containing 10 μg/ml of phenylephrine hydrochloride are shaken with a 5 cm long, cleaned, longitudinally cut strip of rabbit intestine at 37°C. Samples (0.32 ml) are removed at 10-minute intervals starting at zero-time. Phenylephrine disappears from the medium within 30 minutes. Rabbit intestine homogenates are also effective. Inactivation of epinephrine and norepinephrine could also be shown using rabbit intestine and other tissues.

Discussion. Reports in the literature(1-4) invariably state that an initial relaxation period of 2 to 3 hours for the rabbit aortic strip is necessary to acquire sufficient sensitivity and a constant baseline. Use of nitrite to shorten the initial waiting period(8-10) did not serve its purpose. Nitrite might even be objectionable because of the unnecessary additional complexity in design of the experiment.

By application of gentle stretching, initial waiting period can be shortened to 5 to 10 minutes instead of the usual 2 to 3 hours; therefore, deterioration of the tissue need not be feared. Relaxation between 2 doses is also easily obtained by stretching; thus, every 7 minutes a new dose can be applied. In this way the rabbit aortic strip can be used for a variety of purposes as conveniently as the frog rectus abdominis muscle.

By the cumulative method of drug applications, concentrations for responses ranging from minimum to maximum extend approximately to 4– (from 10^{-9} to 10^{-5} M), whereas by the non-cumulative method concentrations extend only to slightly more than one-order of magnitude; in other words, the non-cumulative method gives a much steeper regression line. Therefore, we find the rabbit aortic strip appropriate for precise bioassay of sympathomimetic amines, and for study of their pharmacodynamic features. The described technic could, no doubt, be applied to laboratory diagnosis of pheochromocytoma.

Summary. 1. Gentle stretching of the rabbit aortic strip produces rapid relaxation, and single doses can be applied at intervals as short as every 7 minutes. 2. Use of this preparation for a variety of purposes is described, including a 4-point bioassay of catecholamines and pharmacological studies, as exemplified by potentiation and inactivation.

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Lipid Fatty Acid Composition of Several Areas of the Aorta in Subjects with Atherosclerosis.* (26209)

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It has been suggested(1) that essential fatty acids are required for normal transport and metabolism of cholesterol. According to that hypothesis, when there is a deficiency of those acids cholesterol is esterified with increased amounts of saturated and monoenoic acids and these esters accumulate in the tissues (aorta) producing atherosclerotic plaques. This suggested relationship has prompted a number of studies(2-5) to ascertain if there are any differences in the fatty acid composition of the cholesterol ester fraction of serum and atheromatous plaques. The published data(2-5) are at variance with one another and indicate that the percentage of polyunsaturated fatty acids in the cholesterol ester fatty acids (CEFA) of atheroma may be lower, higher or the same as that fraction in serum. Some of the differences in the earlier data may be due to the fact that the analyses of the cholesterol ester fraction of the aorta were carried out either on wall tissue (intima plus media), plaques plus wall tissue or plaques only. The media accounts for the largest portion of the aorta and the cholesterol ester fraction of that area could dilute out any apparent differences in CEFA composition attributable to the atherosclerotic lesion of the intima. To circumvent some of these difficulties, we recently reported a study(6) in which the CEFA composition of the media (freed from intima), plaque material and serum were compared. It was shown that the cholesterol ester fraction of plaque material had significantly less linoleic acid and more oleic acid than either media or serum. The purpose of the present study was to extend those earlier observations to another area of the aorta (thickened intima) and to obtain data on the triglyceride and phospholipid fractions of several areas of the aorta and serum.

Methods and materials. The tissues, other than serum, were obtained at autopsy from 6 hospitalized male patients, aged 38, 39, 51, 63, 68, and 78 years.† Autopsies were performed within 8 to 12 hours after death and portions of the abdominal aorta removed. The aorta was freed of adherent tissue and its layers carefully dissected along their cleavage planes into adventitia, media and intima. The intima was partitioned, as far as their occurrence permitted, into normal appearing portions, thickened portions or early plaques, and free-lying lipid containing material from far-advanced plaques. Except for one subject, normal appearing intima was not available in sufficient quantities for analysis. Fasting serum was obtained from 9 male patients aged 55-70 years, with occlusive atherosclerosis in a good nutritional status. The tissues were quick frozen in a dry ice acetone mixture, pulverized and initial lipid extractions carried out with 1:1 acetone-alcohol(7) followed by 2:1 chloroform-methanol. Serum was extracted in a similar manner. Cholesterol esters, triglycerides and phospholipids were separated by chromatography on silicic acid(8), with the modification that 100% methanol was used to elute the phospholipid fraction. The minor lipid components were eluted with 25% ethyl-ether in petroleum ether and discarded before elution of the phospholipid. The isolated lipid fractions were interesterified in HCl methanol and methyl esters sublimed according to the method of Stoffel *et al.*(9). Gas-liquid chromatography was carried out as described earlier(6) with a succinate polyester of diethylene glycol as the stationary phase.

Results. Cholesterol ester fatty acid composition (Table I). As in our earlier report (6), media and serum had approximately the

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† The patients suffered from a variety of diseases and had varying degrees of atherosclerosis.

TABLE I. Cholesterol Ester Fatty Acid Composition of Several Areas of Aorta and Serum.

Fatty acid* Chain length carbons	No. double bonds	Area of aorta† Media	Intima thickened	Plaque material	Serum‡
			% total fatty acids		
6 to 12		.6 ± .1§	1.1 ± .8	1.2 ± .6	.9 ± .2
14	0	1.5 ± .3	1.5 ± .8	1.0 ± .3	.7 ± .4
"	1	.4 ± .1	.4 ± .2	.4 ± .2	tr
16	0	15.8 ± .6	17.5 ± 3.6	17.7 ± 1.4	15.9 ± 1.9
"	1	5.5 ± 1.6	6.0 ± 2.0	5.6 ± 1.1	4.6 ± 1.0
"	2	.6 ± .2	.7 ± .4	.5 ± .2	.5 ± .2
18	0	1.7 ± 1.2	2.0 ± .9	1.6 ± .5	2.9 ± .7
"	1	27.6 ± 2.7	36.7 ± 4.3	38.4 ± 3.0	26.2 ± 1.1
"	2	37.9 ± 3.2	28.8 ± 6.4	28.1 ± 1.8	40.8 ± 2.4
"	3	.4 ± .1	.4 ± .1	.6 ± .2	.4 ± .1
20	0	.4 ± .1	.4 ± .1	.5 ± .1	.4 ± .1
"	3	.4 ± .1	.6 ± .5	.7 ± .5	.4 ± .1
"	4	7.2 ± 1.1	3.9 ± 1.7	3.7 ± .9	6.3 ± 1.0
Saturated		20.0	22.5	22.0	20.8
Mono-unsaturated		33.5	43.1	44.4	30.8
Polyunsaturated		46.5	34.4	33.6	48.4

* Represents major fatty acids determined; small amounts of others were also detected.
† Values represent avg of 6 subjects.
‡ Values represent avg of 9 subjects.
§ ± stand. dev.

same CEFA composition. The major acid of the CEFA in those tissues was linoleic acid which accounted for approximately 40% of total acids. Thickened intima (early plaques) and plaque material had almost the same CEFA composition. CEFA of both of those areas of the aorta (thickened intima and plaque material) were different from media and serum, in that they contained significantly less ($P < .01$) linoleic and arachidonic acids and more oleic acid. No significant differences were found between the minor fatty acids of the several tissues.

Triglyceride fatty acid composition (Table II). In the tissue triglyceride fraction, palmitic and oleic acids comprised from 72-

TABLE II. Triglyceride Fatty Acid Composition of Several Areas of the Aorta and Serum.*

Fatty acid Chain length carbons	No. double bonds	Area of aorta Media	Intima thickened	Plaque material	Serum
			% total fatty acids		
6 to 12		1.1 ± .5	1.3 ± .7	2.1 ± 2.1	.8 ± .3
14	0	2.1 ± .8	1.9 ± .5	2.2 ± .7	1.4 ± 1.0
"	1	.5 ± .1	.6 ± .2	.5 ± .3	.2 ± .1
16	0	31.9 ± 2.7	30.9 ± 1.5	31.9 ± 3.0	30.0 ± 3.0
"	1	3.3 ± 1.5	3.4 ± .9	2.9 ± .8	3.8 ± .7
"	2	.4 ± .2	.6 ± .3	.6 ± .5	.3 ± .1
18	0	11.1 ± 4.5	8.2 ± 2.9	7.6 ± 1.5	6.6 ± 1.6
"	1	40.2 ± 5.6	42.1 ± 4.3	40.9 ± 3.6	43.8 ± 2.6
"	2	8.2 ± 1.9	9.8 ± 2.3	10.4 ± 2.0	11.6 ± 2.2
"	3	.4 ± .1	.4 ± .2	.2 ± .1	.4 ± .3
20	0	tr	tr	tr	.3 ± .2
"	4	.8 ± .5	.8 ± .3	.7 ± .2	.8 ± .4
Saturated		46.2	42.3	43.8	39.1
Mono-unsaturated		44.0	46.1	44.3	47.8
Polyunsaturated		9.8	11.6	11.9	13.1

* See footnotes to Table I.

TABLE III. Phospholipid Fatty Acid Composition of Several Areas of the Aorta and Serum.*

Fatty acid		Area of aorta			
Chain length carbons	No. double bonds	Media	Intima thickened	Plaque material	Serum
			% total fatty acids		
6 to 12		.8 ± .4	1.0 ± .4	1.3 ± .5	.6 ± .2
14	0	2.1 ± 1.1	1.9 ± 1.1	2.5 ± 2.0	.8 ± .4
”	1	.7 ± .2	.4 ± .2	.7 ± .6	.4 ± .2
16	0	35.9 ± 1.5	36.2 ± 2.8	31.3 ± 6.2	39.2 ± 3.5
”	1	2.2 ± 1.0	2.9 ± .9	4.2 ± 2.1	2.2 ± .3
18	0	19.8 ± 2.5	16.4 ± 2.3	15.3 ± 4.6	12.6 ± 2.9
”	1	18.9 ± 2.4	22.1 ± 1.5	22.9 ± 3.8	24.5 ± 2.5
”	2	5.6 ± .7	7.4 ± 3.5	8.7 ± 4.1	11.4 ± 2.0
”	3	.5 ± .2	.4 ± .1	.2 ± .2	tr
20	0	.4 ± .2	.3 ± .1	tr	.4 ± .1
”	3	.2 ± .2	.3 ± .1	tr	.5 ± .2
”	4	10.2 ± 2.3	2.6 ± 1.0	1.8 ± 1.1	2.4 ± .5
22	→	2.7 ± 1.7	8.1 ± 2.5	11.1 ± 2.6	5.0 ± 2.5
Saturated		59.0	55.8	50.4	53.6
Mono-unsaturated		21.8	25.4	27.8	27.1
Polyunsaturated†		19.2	18.8	21.8	19.3

* See footnotes to Table I.

† Includes long chain fatty acid fraction, 22 carbons and beyond.

75% of the total fatty acids. Percentages of palmitic and oleic acid were strikingly constant and were in the narrow range of approximately 29-35% and 39-46% respectively. There were only small differences in the remaining triglyceride fatty acids of the several tissues. The most notable was the high level of stearic acid in the media (11.1%). Comparison of CEFA and triglyceride fatty acids indicates that there was a much lower level of polyunsaturated fatty acids in the triglyceride fraction.

Phospholipid fatty acid composition (*Table III*). The phospholipid fraction of the tissues examined was characterized by a high level of saturated fatty acids. This fraction accounted for 50-59% of total fatty acids. The major fatty acids of the phospholipid fraction were palmitic, stearic and oleic acids. The phospholipid fraction of the media had significantly ($P<.01$) more arachidonic acid than that fraction in the other tissues. The phospholipid fraction of the thickened intima and plaque material were similar in fatty acid composition, but contained a greater proportion of long chain fatty acids than either media or serum.

Discussion. Our present results have confirmed our earlier observations(6) and have provided new data regarding fatty acid distribution in the lipid fractions of several different areas of the human aorta. A direct comparison of the data obtained in this study cannot be made with that of earlier reports(2-5) since in the present experiments the aortic media was separated and analyzed independently of the plaques (early and advanced). Of the different lipid fractions analyzed in the several areas of the aorta, the cholesterol ester fraction showed the greatest differences in fatty acid distribution. Distinct differences were noted in CEFA composition of media, serum and plaques (early and advanced). This dissimilarity in CEFA composition of the plaques and serum indicates that there may be a distinct mechanism operating in which cholesterol esters of a more saturated and monoenoic nature are laid down as atherosclerosis progresses (1). It is also of interest that both the early and advanced plaques had approximately the same CEFA composition. The diversity in CEFA composition of aortic media and plaques is particularly noteworthy and provides suggestive evidence that there are distinct differences in the metabolism of the cholesterol ester fraction in those areas of the aorta. The fatty acid distribution in the triglyceride fraction of the several areas of the aorta and serum were similar. It has been observed that there is an increase in the triglyceride and phospholipid content of the intima as atherosclerosis develops, as well as in free and ester cholesterol(10). This, plus the similarity of the triglyceride fatty acid composition in media, serum and plaques suggests that there is an indiscriminate deposition of triglyceride in the aorta. While there were some differences in fatty acid distribution of the phospholipid fraction in the several areas of the aorta and serum, it should be pointed out that that fraction was heterogeneous since it contained lecithins, sphingomyelins and cephalins. The differences in the percentages of the polyunsaturated fatty acids in that fraction of the several tissues studied might be due to the greater abundance of a specific phospholipid. The results of the present study stress the need for further study of the origin and metabolism of the cholesterol ester fraction in the aorta.

Summary. The cholesterol ester, triglyceride and phospholipid fatty acid composition (gas-liquid chromatography) of aortic media, thickened intima and plaque material were determined in 6 human subjects. For comparison, the fatty acid composition of those fractions in serum was determined in 6 subjects (aged 55-70 years) with occlusive atherosclerosis in a good nutritional status. The triglyceride fraction of those tissues and serum were similar in their fatty acid composition. Some slight differences were noted in the fatty acid composition of the phospholipid fractions, most notably in the arachidonic and long chain fatty acids. The cholesterol ester fraction of the tissues studied showed the greatest differences in fatty acid composition. Both early and advanced plaques had significantly less linoleic and more oleic acid in that fraction than serum or media. Media and serum CEFA were similar in their fatty acid composition. The significance of these findings in relation to atherogenesis is discussed.

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Evaluation of Respiratory Mutants for Selecting Compounds for Cancer Chemotherapy Study.*† (26210)

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Many investigators, in their search for systems which might respond similarly to tumor tissue because of some parallelism of metabolism, have attempted to establish a preliminary screen for cancer chemotherapy utilizing organisms which might be induced to preferentially use fermentative metabolism. Studies with anaerobes by Bradner and Clarke(1) as well as by DiPaolo and Rosenfield(2) indicated that results with these sys-

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tems are not actually superior to the results obtained with aerobes.

Respiratory mutants of several yeast strains obtained by incorporating acriflavine (2) into the growth medium differ from the wild type visibly and in their ability to utilize glucose by the oxidative process. It has been theorized that a respiratory mutant which has a change in the mode of utilization of glucose might be an excellent tool for selecting cancer chemotherapeutic agents. Gause(3,4) has reported that mutants with impaired oxidation are selectively inhibited by anticancer preparations and therefore are of value for evaluating new anticancer compounds. Unfortunately, most of the compounds tested by us(2,5) failed to inhibit preferentially the respiratory yeast mutants obtained from several strains of yeast.

More recently Gause has made available through the American Type Culture Collection his *Staphylococcus aureus* and 2 "mutants" derived therefrom (ATCC #13679 to 13681). This paper reports results obtained with a selected group of drugs using our 2 yeast strains and the *S. aureus* strains from ATCC.

Materials and methods. A series of 16 compounds reported as inhibitory on experimental neoplasms and of interest to clinical cancer chemotherapy, was tested by an agar-disc assay method in Petri dishes for their preferential growth inhibition of yeast respiratory mutants and *S. aureus* (ATCC #13680, 13681).

In the yeast study, results obtained with respiratory mutants and the wild type were compared. Mutant colonies were picked at random after being grown on agar medium supplemented with acriflavine. For sensitivity studies the strains of *Saccharomyces cerevisiae* were grown in the medium of Billen and Lichstein(6) and strains of *Saccharomyces carlbergenesis* in medium of Atkin *et al.*(7). Following seeding with a 24-hour-old culture and solidification of the agar, discs containing 100 μg of drug (except for Actinomycin D, 10 μg) were placed on the surface. The dishes were incubated at 28° for 24-72 hours, depending upon the strain. The diameter of each zone of inhibition was measured in millimeters.

TABLE I. Results of Inhibition Tests Using a Selected Series of Compounds.

Drug	Yeasts	*Staphylococcus aureus*
	Inhibitory index*	
5-Fluorouracil	35/50	40†/34
5-Fluorodeoxyuridine	23/20	25/22
N,N′,N″-Triethylene thiophosphoramide	17/20	0/0
Methyl bis (β chloroethyl) amino	28/30	0/0
A-methopterin	45/45	20/5
8-azaguanine	44/10	0/0
Azaserine	19/20	16/16
Actinomycin D	0/0	24/38
Diethylstilbestrol	0/0	20/18
Nitromin	0/0	0/15

* Zone of inhibition in mm of parent/zone of inhibition in mm of mutant.

† Partial zone.

For the *S. aureus* tests the cells were grown for 18-24 hours at 37° in brain-heart infusion broth. *S. aureus* (ATCC #13679) was diluted 1:100 while the "mutants" were diluted 1:10. One ml of cell suspension was mixed with brain-heart infusion agar. Discs identical to those used with the yeast were placed on the hardened agar. After 24 hours' incubation at 37°, zones of inhibition were evaluated as above.

Results. The following compounds, at the concentrations used, did not inhibit any of the yeasts or staphylococci: estrone, urethan, 6-chloropurine, hydrocortisone, 6-mercaptopurine and AB-103 (Benzyl N-bis(ethylenimido)phosphorocarbamate). The results for yeast are grouped together because neither yeast strain nor mutant was more sensitive than any other. One compound, 5-fluorouracil, selectively caused greater inhibition of yeast mutants and staphylococci "mutants" than the parental types.

The remaining yeast results show the same degree of inhibition for mutants and parents except for 8-azaguanine which preferentially inhibited the parental types. The staphylococcal "mutants" were selectively inhibited by nitromin, relatively more inhibited by actinomycin D and less inhibited by A-methopterin than was the parent. No differences were noted with the remaining compounds.

Discussion. The results presented can not be considered as substantiating evidence endorsing the use of this test as a primary screen for cancer chemotherapy. Thus far, additional results with about 700 compounds including 150 antibiotic beers have been obtained using several yeast species and respiratory mutants obtained from them. In those cases in which occurred preferential inhibition of the mutants and not of the parents, the compound was tested in several *in vivo* experimental animal tumor systems. No compound was an effective tumor inhibitor. The yeast study did not indicate any class of compound as being particularly inhibited.

Additional study of Gause's staphylococci (ATCC #13679-13681) is not contemplated. Gause claims that strain 13680 and 13681 are respiratory "mutants" produced by ultraviolet irradiation of *S. aureus* 13679. While the present study verified his findings that these strains have a Q_{O_2} of approximately 48 and 42% that of the parent culture, the parent strain and the "mutants" were found to differ in several respects. Visibly, 13679 produced typical golden colonies while the substrains produced pink colonies. A typical *S. aureus* enzyme, coagulase, was not present in the substrains. Further differences were found in the growth characteristics of "mutants" and the parent strain. The parent grew overnight with 7.5% sodium chloride in the medium, or in tryptose-glucose broth and produced acid but no gas from mannitol. The substrains grew poorly after one week with 7.5% sodium chloride in medium, after 3 days in tryptose-glucose broth, and produced neither acid nor gas from mannitol after one week. Neter *et al.*(8) found large amounts of Rantz antigen in the supernates of the parent strain in contrast to the mutants. Furthermore, only the supernates of the parent strain inhibited agglutination by *S. aureus* and *L. monocytogenes* anti-sera of erythrocytes modified by either *B. subtilis* or *L. monocytogenes* antigens.

Since mutants obtained by ultraviolet irradiation are usually considered to be altered at a single enzymatic locus, it seems unlikely that a change in a single enzyme should have led, in this instance, to such extensive differences between parent and substrains. Strains 13680 and 13681 probably represent contaminants of the original culture of *S. aureus* 13679.

Summary. Respiratory deficient mutants of yeast, obtained by incorporating acriflavine into the growth medium, as well as *S. aureus* "mutants" obtained from ATCC were used for testing cancer chemotherapeutic agents. Although there is a feasible theory that supports the use of a respiratory mutant with a change in the mode of utilization of glucose, the procedure described appears to be of limited value. Examination of the *S. aureus* mutants (ATCC #13680-13681) from Gause lead to doubt of their authenticity.

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The Properdin System in Mice.* (26211)

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Properdin, a naturally occurring serum protein described by Pillemer *et al.*(1) has been implicated as a factor of importance in the natural resistance of man and other mammalian species to infection. Properdin, in conjunction with Mg^{++} and all 4 components of complement constitutes the properdin system. Evidence(1-7) indicates that this humoral system participates in destruction of certain bacteria and protozoa, inactivates certain viruses and is involved in resistance or susceptibility of experimental animals to infection. "Properdin by itself is not bactericidal, and it has been shown to be without bactericidal effect, even in concentrations 100 times its concentration in serum"(8). Among the serums of a variety of species assayed for properdin activity, the laboratory rat shows highest activity and the laboratory albino mouse ranks second with a serum properdin level of 10-20 units/ml(1).

The absence of bactericidal activity of normal mouse (albino *Mus musculus*) serum has been reported(9-11). The apparent inconsistency of high properdin levels but absence of serum bactericidal activity can be resolved if one recalls that mouse serum is deficient in one or more components of complement (C′)(12-14). Since all fractions of C′ are reported to be necessary for properdin activity, this lack could account for the failure of mouse serum to be bactericidal.

The following studies were based on 2 hypotheses. First, if the properdin system is, indeed, concerned with humoral mechanisms of host resistance and susceptibility, then parenteral injection of C′ into the mouse, which has high properdin levels, may alter the animal's ability to withstand bacterial infection. Second, addition of C′ to mouse serum which is lacking certain C′ components but is high in properdin content, should result in demonstration of bactericidal activity *in vitro*.

Materials and methods. 1. *Bactericidal test.* The procedure for testing *in vitro* bactericidal activity was to add a 0.2 ml aliquot of a suitable dilution of *Klebsiella pneumoniae* or *Salmonella typhimurium* to mouse serum or a mixture of mouse serum and guinea pig serum (C′) to give a final volume of 2.0 ml. Inocula of 1,000 to 100,000 organisms/ml were used; aluminum capped tubes containing these inocula were incubated in a 37°C water bath for 1.5 hours. Pour plates were then prepared using 1 ml aliquots of appropriate dilutions of the incubated serum-organism suspension. These plates were then incubated at 37°C for 24 to 36 hours and colony counts made.

2. *Complement.* Fresh guinea pig serum, which has the highest C′ activity of any laboratory animal(12) served as the source of C′ throughout these studies.

Results. Preliminary work showed that when groups of mice were subjected to total body x-irradiation, a procedure well known to produce infection and bacteremia of enteric origin, and then treated with C′ (guinea pig serum), antibiotics, or a combination of these materials, that increase in survival resulted only among antibiotic treated animals. Table I summarizes these treatments and results obtained. Although injections of complement failed to increase survival of x-irradiated mice, the results could not be taken as evidence that this failure was expressing the failure of C′ to interact with properdin since mouse serum properdin levels have been reported to be markedly decreased following x-irradiation exposure(15).

Experiments were, therefore, conducted to determine if C′ (0.1 or 0.2 ml guinea pig serum) injected subcutaneously could reduce mortality by activating the properdin system. The test system chosen were normal mice

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TABLE I. Effect of Antibiotic and/or Complement Treatment* on Survival of Irradiated Mice.

X-radiation dose (r)	30-day mortality			
	Saline (stress control)	Complement	Antibiotic mixture	Antibiotics and complement
	%	%	%	%
350	4/26—15	3/20—15	1/20— 5	9/20—45
400	10/23—43	12/20—60	4/20—20	9/20—45
450	24/25—96	17/20—85	12/20—60	13/20—65

* Treatment consisted of daily inj. of 0.1 ml C′ intraper., 5 mg streptomycin and 5000 u penicillin, or combinations thereof for 2 wk and thereafter every other day for 10 days; saline and antibiotics inj. subcut., nuchal area (0.1 ml).

challenged with either *K. pneumoniae* or *S. typhimurium*. The results presented in Table II record the failure of C′ to increase survival of mice infected with *K. pneumoniae*; similar results were obtained with *S. typhimurium*. Both organisms were shown to be susceptible to *in vitro* bactericidal activity of either rat or rabbit serum. Nevertheless, these results cannot be accepted as evidence that C′ failed to activate the properdin system since with this *in vivo* system it is impossible to exclude the interaction of other intrinsic mechanisms which might be contributing to the observed results, *e.g.*, inactivation of either C′ or properdin.

In an effort to evaluate this latter possibility, an isolated *in vitro* system was employed to test the second working hypothesis; *i.e.*, that addition of C′ to normal mouse serum would restore bactericidal activity *in vitro*. Table III records results of a typical experiment. It appears at first glance that C′ failed to render mouse sera bactericidal against the test organism, *S. typhimurium*. However, upon further analysis it is apparent that mouse serum, in the concentrations employed, is anticomplementary (compare line 3 with line 6). Therefore, the conclusion that C′ fails to restore bactericidal activity in the mouse may be unjustified. That is, some substance(s) in mouse sera may have destroyed the added C′ which was to react with the properdin present in the system and result in bactericidal activity.

TABLE II. Effect of Complement Injections on Survival of Mice Infected with *Klebsiella pneumoniae*.

Group*	Complement treatment	Mortality, %
I	No treatment (organism challenge control)	52
II	0.1 ml subcut. (nuchal area) injections of C′ twice daily for 1 wk; first C′ inj. preceded challenge by 12 hr	48
III	0.2 ml *idem*	64
IV	0.1 ml subcut. (nuchal area) injections of C′ twice daily for 3 days prior to and 7 days following challenge	60
V	0.2 ml *idem*	56

* Each group contained 25 animals.

TABLE III. Failure of An Experiment Designed to Test Bactericidal Activity of Mouse Serum against *Salmonella typhimurium** Due to Anticomplementary Activity.

Tube	PSS (ml)	C′ (ml)	Mouse serum‡ (ml)	Bactericidal activity† (samples)				
				1	2	3	4	5
1	1.8	0	0	m	m	m	m	m
2	1.7	.1	0	m	m	m	m	m
3	1.3	0.5	0	+	+	+	+	+
4	0	0	1.8	m	m	m	m	m
5	0	.1	1.7	m	m	m	m	m
6	0	.5	1.3	—	—	—	—	—

* Prior to incubation a 0.2 ml broth suspension of organisms was added to the test system to give an initial avg concentration of 880 organisms/ml with a range of 780 to 920 organisms/ml.

† A sample was negative (—) if there was no reduction of initial number of organisms or if multiplication (m) took place. Positive (+) bactericidal activity was reported if bacterial count was reduced by at least 50%.

‡ Each sample consisted of pooled serum from 5 mice.

An effort was made, therefore, to determine maximum amount of complement (guinea pig serum) which was not bactericidal in order that a maximum concentration of complement could be added to this *in vitro* system. At the same time maximum amount of mouse serum which would lack anticomplementary activity under the test conditions was determined. It was found that 0.1 ml of pooled undiluted guinea pig serum in the

TABLE IV. Failure of Complement To Render Mouse Sera Bactericidal.

Tube	C′ (ml)	Serum* (ml)	PSS (ml)	No. of surviving organisms†	
				K. pneumoniae	*S. typhimurium*
1	.1	—	1.7	17,100	11,500
2	.1	.4 (mouse)	1.3	16,500	10,600
3	.2	—	1.6	13,200	2,300
4	.2	.4 (mouse)	1.2	14,300	3,500
5	—	.4 (")	1.4	18,000	10,800
6	—	—	1.8	17,600	10,200
7	—	.4 (rat)	1.4	2,100	0
8	—	.4 (rabbit)	1.4	3,400	0

* Mouse serum sample consisted of pooled serum from 5 mice.

† Prior to incubation a 0.2 ml broth suspension of organisms was added to each tube to yield an initial concentration of approximately 17,000 *K. pneumoniae* organisms/ml or 10,000 *S. typhimurium* organisms/ml.

final test volume of 2.0 ml was uniformly non-bactericidal. Likewise, from a pool, 0.4 ml of undiluted mouse serum in a final volume of 2.0 ml (final dilution 1:5) was the largest amount which could be routinely used without exhibiting anticomplementary activity.

With this information a number of experiments were carried out to determine whether a mixture of these concentrations of C′ and mouse serum would yield bactericidal activity. The experimental results were uniformly negative. Table IV presents results of a typical experiment. Mouse serum exhibits no bactericidal activity (tube 5) whereas both control rat and rabbit sera are markedly bactericidal for either *K. pneumoniae* or *S. typhimurium* (tubes 7 and 8). Addition of 0.1 ml of C′ (guinea pig serum) failed to restore bactericidal activity to mouse serum (tube 2). Guinea pig C′, 0.2 ml, possessed bactericidal activity (tube 3) and apparent restoration of such activity to mouse serum (tube 4) must be attributed to the bactericidal activity of the added C′.

Discussion. We have been unable to adduce evidence in support of either of the proposed working hypotheses. Injection of C′ into the laboratory albino mouse failed to alter ability of the available strain of animals to withstand bacterial infection, and addition of C′ to mouse serum failed to induce bactericidal activity *in vitro*.

The experimental design had inherent limitations and certain factors require additional comment. Questions to be considered are: 1. Was amount of C′ added in these tests sufficient for activation of the mouse properdin system? C′ assays revealed that the pooled guinea pig sera (the source of C′ in these experiments) used never had a titer of less than 330 units/ml. Therefore, in the *in vivo* studies, mice received either 33 or 66 units of C′ twice daily. With *in vitro* bactericidal tests, at least 16.5 units were added per tube. It is of interest that Kornfeld(16, 17) reported that addition of 0.2 ml of 1:5 or 1:10 diluted guinea pig serum to 0.3 ml of serially diluted mouse serum would yield bactericidal action with normal mouse serum. 2. In the *in vivo* studies, were succeeding injections of C′ inactivated by formation of anti-complement antibody from preceding injections? Since whole guinea pig serum was utilized as the source of complement, antibody would be expected to develop. Whether rate of formation was rapid enough to yield significant amounts of antibody resulting in partial or complete inactivation of C′ is open to speculation. No overt signs of untoward reactions to C′ injections were observed among the experimental animals. 3. The routine procedure utilized in testing bactericidal activity always results in a final dilution of 1:5 for mouse serum. Could the dilution factor be critical in the observed failure to obtain bactericidal activity? Undoubtedly this dilution may have decreased bactericidal activity. It should, however, be noted that rat and rabbit control sera, when diluted 1:10, still retained bactericidal activity.

Although the data presented cannot be taken as definitive evidence that the properdin system is not significant in the natural defenses of the mouse, the results obtained do not contribute evidence to support the suggested importance of this system.

Summary. Injection of complement (guinea pig serum) into the laboratory albino mouse failed to alter capacity of these animals to withstand parenterally induced bacterial infection with *K. pneumoniae* or *S. typhimurium.* Addition of complement to mouse serum failed to induce bactericidal activity *in vitro.*

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Total Exchangeable Potassium in Systemic Lupus Erythematosus with Reference to "Triamcinolone Myopathy".* (26212)

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Muscular weakness and atrophy have been described following prolonged use of adrenal steroids, particularly triamcinolone(1,2). This has been referred to as triamcinolone myopathy(3). Histologic changes in the muscles of such patients were relatively minimal and consisted of scattered areas of muscle vacuolization and proliferation of sarcolemmal muscle. Increased creatine synthesis and decreased ability to store this substance have been suggested as a cause of the creatinuria observed in these patients. Potassium loss also has been considered as a possible pathogenic factor although serum potassium levels have been normal in triamcinolone myopathy.

To elucidate the effect of adrenal steroids on total body potassium stores, 2 groups of patients with systemic lupus erythematosus (SLE) were studied. One group of patients had a mild form of the disease and received only symptomatic therapy with salicylates and antimalarials. The other group was severely affected and had been treated with various adrenal corticoids.

Total exchangeable potassium (Ke) was determined according to the method of Corsa *et al.*(4). Radioactive potassium (K-42) was given orally in tracer doses of 300-500 μc.‡ The specific activity of K-42 was .2-.4 mc/mg so that only a minute amount of stable inorganic potassium was administered. Radioactive potassium (K-42) and stable potassium (K-39) concentrations were deter-

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‡ Obtained from Abbott Labs., Oak Ridge, Tenn., under AEC license.

TABLE I. Total Exchangeable Potassium Values in (1) A Group of Female Controls, (2) Female Patients with Systemic Lupus Erythematosus on Non-Steroid Therapy, and (3) Female Patients with Systemic Lupus Erythematosus on Steroid Therapy.

Age	K^{39}, meq/l urine	K^{42}, μc/l urine	Ke, meq	Ke, meq/kg	
	Female controls				
32	32	7.53	2045	36.5	
68	62	12.00	1520	30.8	
51	48	6.02	2300	39.7	
63	29	3.53	2340	29.1	
39	8.5	1.22	1980	31.2	
53	42	5.07	2300	36.2	
Mean Ke 2081 ± 311* meq or 33.9 ± 4.1* meq/kg					
	Female S.L.E. non-steroid therapy				
43	28	4.56	2153	49.3	
29	30	3.79	2678	32.2	
27	27	4.18	2179	38.1	
32	8.4	1.28	1870	35.6	
47	14	1.77	2220	31.1	
Mean Ke 2220 ± 291 meq or 37.3 ± 7.3 meq/kg					
	Female S.L.E. on steroids				Therapy
51	19	3.8	1742	20.8	Steroids 1 yr; triamcinolone, >4 mg/day/4 mo, 4 mg/day/2 mo
43	51	10.5	1370	26.2	Dexamethasone, 1 mg/day/4 mo
41	9.5	2.18	1730	29.7	Hydrocortisone, 50 mg/day/1 mo; triamcinolone discontinued for 2 mo; cortisone discontinued for 1 mo
29	22.5	5.2	1947	36.8	Medrol, 12 mg/day; Diuril, 1.0 g/day
38	58	11.9	2142	44	Triamcinolone, 40 mg/day/2 mo; cortisone, 100 mg/day/2 mo
Mean Ke 1786 ± 288 meq or 31.5 ± 9.1 meq/kg					

* Stand. dev.

mined after complete exchange had taken place (24 hours) and spot samples of urine at 25, 29 and 30 hours were examined to be certain that complete exchange had taken place.

Our mean normal values and their standard deviations were:

3530 ± 873 meq or 46.5 ± 8.4 meq/kg for 6 male control subjects and

2081 ± 311 meq or 33.9 ± 4.1 meq/kg for 6 female control subjects.

Total exchangeable potassium (Ke) values of the patients with systemic lupus erythematosus on non-steroid therapy and those on steroids do not differ significantly from those of the normal control group (Table I).

Control group *vs* SLE on non-steroid therapy $0.3 < p < 0.4$

Control group *vs* SLE on steroid therapy $0.5 < p < 0.6$

SLE on steroids *vs* SLE on non-steroid therapy $p = 0.3$

Patient B.D. had 3 separate determinations done over a period of one year during which time she received triamcinolone, cortisone, hydrocortisone and symptomatic therapy. At time of the second test, as she was much weaker, cortisone was added to triamcinolone to ascertain whether this might reverse the "myopathic" action of triamcinolone. There was no clinical improvement until this latter steroid was stopped (Table II). The changes in Ke are not striking and could not be attributed to triamcinolone, since such changes should have been apparent at time of first Ke determination, after 9 months of triamcinolone therapy. The variations in total exchangeable potassium are more likely due to progression of the disease.

Urinary potassium excretions in all the

TABLE II. Serial Total Exchangeable Potassium Values in a Patient (B.D., 32-Year-Old Female) with Systemic Lupus Erythematosus on Various Steroid Regimens.

	Wt, kg	Ke, meq	Ke, meq/kg	Urinary K^{39}, meq/l	Therapy
Dec. 1958	58	1995	34.2	27	Triamcinolone, 8-32 mg every day for 9 mo; marked proximal muscle weakness
Jan. 1959	58	1464	25.1	48	Triamcinolone continued plus cortisone, 50 mg every day; K Triplex, 6 g every day for subsequent 5 wk; exceedingly weak; no change in clinical picture
March 1959	58	1731	29.8	9.5	Triamcinolone DC—2 mo; cortisone DC—1 mo; hydrocortisone, 50 mg every day for 1 mo, strength normal

patients with SLE did not vary from those of the normal control subjects.

It is concluded that total exchangeable potassium in patients with SLE did not vary significantly from that of normal female control subjects and that triamcinolone as well as other corticosteroids did not appear to have a demonstrable effect on it.

Summary. Total exchangeable potassium, using K^{42}, was determined in a group of female control subjects and 2 groups of patients with systemic lupus erythematosus. One of these groups was receiving adrenal cortical steroids including triamcinolone, the other salicylates and antimalarials only. Serial determinations were also done on one patient receiving long term steroid therapy. No significant differences in total exchangeable potassium values could be demonstrated among the 3 groups, and steroid therapy, including triamcinolone, did not appear to have an effect on total exchangeable potassium.

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Erythropoietic Stimulating Factor Production in Pubescent Mice after a Single Exposure to Hypoxia. (26213)

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After chronic exposure of mature animals to hypoxia, the first morphological evidence of the resulting polycythemia is reticulocytosis which becomes maximal on the third or fourth day after initial exposure(1). After that, the number of mature erythrocytes gradually increases for 7 or 8 weeks(*,2). The results of the present experiment show that BDF1 mice have a reticulocytic response greater than baseline value after puberty when subjected to a single hypoxic exposure, but do not have this capacity before puberty. Furthermore, if production of erythropoietic stimulating factor (ESF) is accepted as the prerequisite to the reticulocytosis, it can be said that the endocrine changes at puberty are concomitant with the animals' ability to increase production of ESF.

Methods. The experiment was carried out

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* Unpublished results.

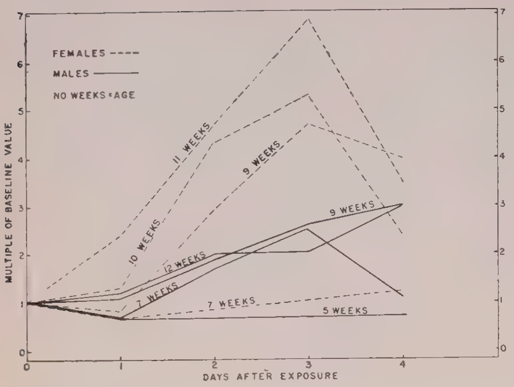

FIG. 1. Increase in reticulocytes in BDF1 mice after a single 6 hr exposure to 22,000 ft.

on BDF1 mice of age groups that included not only the age of puberty but also short intervals before and after that period. They had free access to rat chow and water. Their weights varied from 16-27 g depending on their age grouping. The hypoxic exposure period was 6 hours at 22,000 feet. The animals were subjected to the hypoxic incident in groups of 7 of similar sex and age, placed in a large glass desiccator which served as the decompression chamber. Air was drawn through the chamber continuously as such a rate that its total volume of 5500 ml was changed every 72 seconds; at this rate, rebreathing of expired air is negligible. After the hypoxic exposure, cardiac punctures were made on the 1st, 2nd, 3rd, or 4th day for hematocrit and reticulocyte determinations. A different group was used for each day and each group was used only once. Hematocrits were done by the micro method and the counts were made by the wet-staining technic using methylene blue but without counterstaining. As the determined value of each parameter the mean of each group of 7 animals was tabulated.

To eliminate the differences which occur in reticulocyte counts due to variations in personal interpretations, the counts were all done by one person. The smear used in the counting was thin enough so that random fields could be used over the entire slide; counting errors due to clumping on certain sections of the slide were thereby minimized. Types O-IV cells, which include all earliest to latest forms were enumerated. All of the counting was done by phase microscopy using a "dark-medium" objective. This technic makes it possible to observe protein differences in the reticulocyte as it matures. Thus, the earliest, normoblastic form has a definite pink cytoplasm that gradually fades as the cell approaches maturity.

Baseline values were determined using similar animals without exposure to hypoxia.

Results. Fig. 1 and Table I show the variation in number of reticular cells with time after a single hypoxic exposure of pubescent BDF1 mice. This experiment demonstrates that the male and female of this species have increased capacity for erythropoietic response after reaching the age of 6 weeks, the approximate age of puberty.

The character of the delayed reticulocytic response of the acutely hypoxic, mature females was the same as that of female rats chronically exposed to hypoxia in an earlier experiment(1). The reticulocytosis caused by production of ESF is followed by full

TABLE I. Change in Reticulocyte Counts in Pubescent BDF1 Mice after Exposure to Hypoxia.

	Reticulocytes/1000 erythrocytes				
Age (wk)	Baseline	1st day	2nd day	3rd day	4th day
			Females		
7	12.3 ± 3	9.1 ± 3	—	12.4 ± 6	13.1 ± 2
9	10.7 ± 4	10.6 ± 5	32.1 ± 20	52.6 ± 18	44.3 ± 17
10	11.1 ± 7	12.7 ± 5	46.9 ± 16	49.1 ± 3	26.5 ± 12
11	11.0 ± 6	26.3 ± 13	52.8 ± 14	75.8 ± 20	37.9 ± 10
			Males		
5	141 ± 50	95.2 ± 18	90.2 ± 6	99.3 ± 20	95.4 ± 14
7	43.1 ± 12	38.8 ± 19	72.7 ± 21	110 ± 33	49.6 ± 15
9	32.2 ± 8	33.8 ± 8	62.4 ± 26	85.3 ± 21	96.1 ± 31
12	10.7 ± 6	12.3 ± 8	22.6 ± 7	23.3 ± 8	31.0 ± 9

Each value is the mean of 7 animals, S.E. by the method of R. B. Dean and W. J. Dixon, *Anal. Chem.*, 1951, v23, 636.

polycythemia in animals (†,2) after chronic exposure to hypoxia for several weeks. Since the reticulocytosis in the present experiment is equivalent to that of chronic exposure, it may be concluded that ESF was produced in the animals during the 6 hour residence at 22,000 feet.

There were no significant changes from baseline values in the mean hematocrits of the heptal groups during the 4 days following acute hypoxic exposure.

Discussion. Gordon and Charipper(3) pointed out that there were definite erythropoietic effects following injection of gonadotropic hormones in rats. Their research showed that the adenohypophysis was not involved since androgens had positive effects even on hypophysectomized animals, while estrogens produced an intensification of the marrow hypoplasia of such animals. This experiment further indicates that gonadotropic changes occur at the same time that the animal has increased capacity to respond to hypoxia by production of ESF.

In measuring production of ESF by means of reticulocytosis, other stimuli that produce it must be ruled out. Radiation(4) and nerve stimulation(5) produce a degree of immediate mobilization of reticulocytes rather than the true physiologic response of ESF. Acapnia, resulting from the hyperventilation in an altitude experiment such as this, could be interpreted as the cause of erythropoietic activity. However, bone marrow activity also results when carbon dioxide tension is at normal and elevated levels(6). Grant and Root(7) have concluded that there is no demonstrable relation between erythropoietic activity and carbon dioxide tension.

Plum(8) has pointed out that reticulocytosis can be caused by injection of various chemicals and other causes, but these responses cannot be "considered pure in the same sense that other factors can be left completely out of consideration." In our experiment, since no extraneous factors were introduced, it was concluded that the reticulocytosis was due to the true physiologic stimulation of ESF.

Summary. These results indicate that ESF is produced in BDF1 mice after a 6-hour exposure to 22,000 feet. It is further demonstrated that these mice have augmented response to hypoxia at time of increased endocrine function at puberty.

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† Unpublished results.

A Persistent Hog Cholera Viremia in Young Pigs.[*] (26214)

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When an attenuated hog cholera virus is given susceptible pigs, expectations are that a viremia will develop within 2 weeks after inoculation, followed shortly by clearance of virus from the blood as the pigs develop immunity. During epidemiological studies on hog cholera, some young pigs did not clear virus from their blood as expected. This persistent virus strain has been found to be cytopathogenic(1) in contrast to its ances-

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[*] The authors acknowledge with appreciation support from the Office of Naval Research.

TABLE I. Influence of Age and Immune Status of Mother on Growth and Persistence of Hog Cholera Virus in Pigs.

Age	Status of mother	Growth results*	Presence of virus in blood		
			0 wk	1 wk	>3 wk
6 wk	Immune†	25/25	0/25	0/6	0/25
6 ”	Non-immune	4/25	0/25	25/25	21/25
3 mo	” ”	25/25	0/5	25/25	0/25

* Numerator indicates pigs that gained more than a pound daily or showed virus in blood. Denominator is No. of pigs tested.

† Six of these pigs came from non-immune mothers but were given colostrum from immune mothers.

tral virulent strain A maintained by serial passage in pigs and the derivative modified by rabbit passage(2) that produced this persistent strain, which were not. It seemed worthwhile to report this phenomenon of persistent viremia as well as characteristics found for the persistent virus.

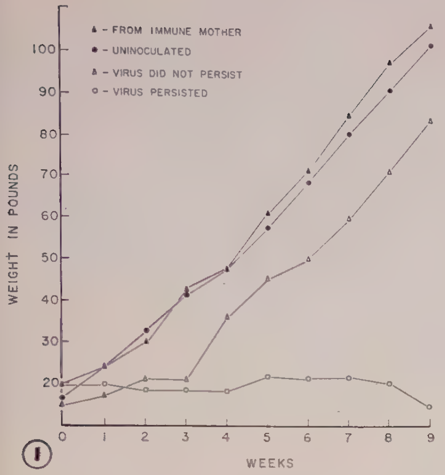
Materials and methods. Litters of pigs were obtained from a disease-free herd maintained by the Institute. After weaning, 25 pigs 5 weeks of age and 25 pigs 11 weeks of age were placed in isolation and 1 week later, each was given 1 ml of a 10% spleen emulsion prepared from a rabbit infected with modified strain A hog cholera virus. From another herd, an additional group of 19 pigs whose mothers all had been immunized against hog cholera was inoculated in a similar manner at the age of 6 weeks. Also, 1 day prior to inoculation of virus, six 6-week-old pigs from the Institute herd were inoculated intraperitoneally with 125 ml of colostrum obtained from hog cholera immune mothers. Uninoculated pigs, 19 from the hog cholera immune herd and 6 from the disease-free herd, were kept as controls. For all of the pigs in these tests, temperatures were taken daily and weights were recorded each week. Results are presented in Table I.

Blood was taken from all pigs before inoculation and at weekly intervals thereafter and tested for presence of virus by inoculation into other susceptible pigs. Also, when they became moribund portions of spleen were removed from each, stored under dry-ice refrigeration, and later titrated for virus content. For comparison, after they became moribund, spleens were obtained from 5 pigs that had been inoculated with virulent hog cholera in the usual manner. Tenfold dilutions of spleen emulsion supernatants that ranged from 10^{-1} to 10^{-10} were prepared and pigs were inoculated to determine the greatest dilution that would produce hog cholera. Hog cholera was determined by clinical response and autopsy findings.

Results. Uninoculated pigs showed average daily gains of weight greater than 1.0 lb per day. Pigs inoculated when 3 months of age also showed average daily weight gains of greater than 1.0 lb daily when calculated over a period of 4 weeks, although weight gains, if any, during the second week after inoculation were slight. Inoculated pigs from immune mothers and those given colostrum from immune sows grew as well as uninoculated controls. In contrast, 21 of the 25 pigs from non-immune mothers that were inoculated when 6 weeks of age either gained less than 1.0 lb daily or showed no gain. Typical growth of the pigs is shown in Fig. 1.

After inoculation of virus, all pigs from non-immune mothers, whether 3 months or 6 weeks old, showed an elevated temperature that began 3 or 4 days after inoculation (Fig. 2). In all of the older pigs, as well as the few younger ones that grew well, temperatures remained elevated for 3 to 5 days, then returned to the normal range. In contrast, the younger pigs that failed to grow showed temperatures that ranged above 105°F for 3 to 5 days and then fell to a level around 104°F. They continued to eat, however, and showed normal activity until a few days before death, which occurred at various intervals from 6 to 17 weeks after inoculation. Only 3 of these 21 pigs that showed elevated temperatures and failed to grow during the first 4 weeks after inoculation appeared to recover. Their temperatures returned to normal range, virus disappeared from their blood, and they grew.

Modified virus was present in the blood of all pigs from non-immune mothers that were tested 1 week after inoculation. Tests 3 or 4 weeks after inoculation showed no virus in the blood of the older pigs or in the younger pigs that grew, but virus was recovered from the blood of pigs that failed to grow. This



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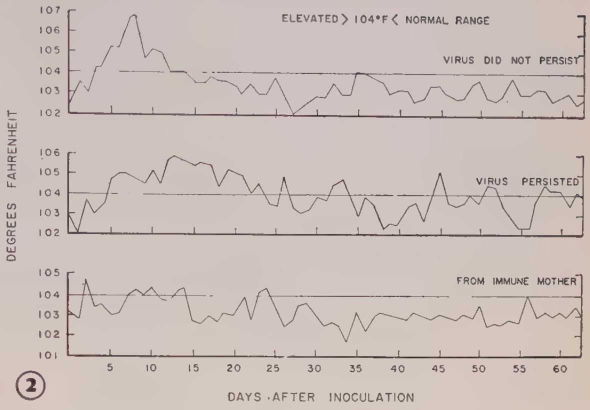

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FIG. 1. Growth of pigs given hog cholera virus.
FIG. 2. Temperatures of pigs given hog cholera virus.

recovered virus had changed its character from modified to virulent. Additional pigs inoculated with this persistent virus, recovered from the spleen of stunted pigs, showed a shorter incubation period and died in less time than pigs given virulent virus maintained in the usual manner. Virus was not recovered either 1 week or 4 weeks after inoculation from the blood of pigs from immune mothers or those inoculated with colostrum.

As death approached in the stunted pigs, their temperatures occasionally rose abruptly to levels above 106°F and they developed paralysis of the hind limbs. After onset of these signs, pigs died within 2 or 3 days. Suspensions of spleen from 10 pigs with these signs infected susceptible pigs, 3 at a dilution of 10^{-7}, 3 at 10^{-8} and 4 at 10^{-9}. In contrast, spleen suspensions from 5 moribund pigs that had been inoculated with virulent virus in the usual manner infected 2 at a dilution of 10^{-4}, 3 at 10^{-5} and none at 10^{-6}.

Discussion. According to usual concepts, modified hog cholera virus should be present in the blood of a pig shortly after inoculation and then disappear after 2 to 3 weeks. When pigs 3 months of age or older were inoculated, usual expectations were met and virus was not demonstrated in the blood after 3 weeks. But younger pigs that were infected when 6 weeks of age generally failed to clear virus from their blood unless, either by suckling or by intraperitoneal inoculation, they received colostrum from mothers immune to hog cholera.

Viral persistence seems less complex if it is considered in relation to sites in which virus can localize after an initial attack. Viruses that persist are those types that localize in superficial tissues and, as a consequence, can be less affected by antibodies. A typical illustration is that of infectious canine hepatitis virus that initially produces viremia but, after antibodies develop, can no longer be demonstrated in the blood, but can be recovered and demonstrated for months afterward in the urine(3). Age of the dog apparently has little effect upon ability of this virus to persist. Viruses of the psittacosis-lymphogranuloma group, perhaps certain enteroviruses, and, of course, herpes virus all have their primary sites of multiplication in superficial tissues and such viruses have been found to persist for variable periods of time. Because fewer viruses produce persistent viremia, it has been suggested that in order to persist in blood a virus must be transmitted either to an unborn or to a newly born individual and reach accord with it without development of antibodies by the host(4). Such a condition of immunological tolerance has been described for lymphocytic choriomeningitis virus(5) transmitted *in utero* and seems to be the epidemiological method used by this virus to reach new hosts

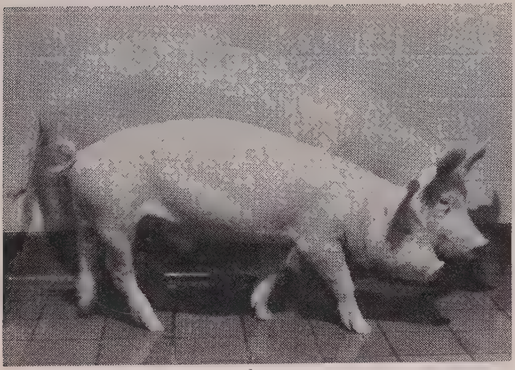

FIG. 3. These 2 pigs were littermates and were the same size when infected with hog cholera virus at 6 wk of age. Larger pig was given colostrum from immune swine while smaller was untreated. Smaller pig shows stunting effect produced by persistent virus.

(6). A similar immunological tolerance has been shown for pigs, but at an older age. Since hog cholera virus also transmits *in utero*(7), the ability to do so in combination with viral persistence may explain the obscure epidemiology of this virus.

Immunological tolerance of hog cholera virus by young pigs was characterized by their inability to overcome the virus, yet to survive and, except for stunting, appear active while tremendous quantities of virulent virus continued to circulate in their blood (Fig. 3). Virus which had persisted in this manner developed several interesting characteristics: (1) reversion to a greater virulence than that possessed by the ancestral strains, (2) ability to produce relatively enormous quantities in the blood, and (3) marked cytopathogenicity in tissue culture, a property not possessed by the ancestral strains. Either markedly enhanced or strikingly diminished pathogenicity is thought to be a mutation; it has been suggested that by appropriate control of environment, mutants can be selected (8). Under this definition, both the production and characteristics exhibited by this persistent hog cholera virus would seem to indicate that it is such a mutant, while the effects it produces in tissue culture would support this supposition.

Summary. A persistent hog cholera viremia was produced in pigs from non-immune mothers that were 6 weeks of age, but not in pigs that were 3 months of age or in pigs from immune mothers that were 6 weeks of age. Although a similar condition of immunological tolerance has been reported for other animals, it was produced either *in utero* or shortly after birth. These pigs, therefore, appear unusual because persistence of virus was established at an older age. Pigs with persistent viremia failed to grow and showed elevated temperatures but survived for periods from 6 to 17 weeks. During this interval, persistent virus reverted to a fully virulent state. When they became moribund, virus titers of spleens from stunted pigs ranged from 10^7 to 10^9 in contrast to 10^5 found in spleens from moribund pigs infected with virulent virus in the usual manner. The ability of hog cholera virus to transmit *in utero* and to persist in pigs after birth may have epidemiological implications.

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Propagation of Hog Cholera Virus in Tissue Culture.* (26215)

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Cultivation in tissue culture of a cytopathogenic strain of hog cholera virus would be useful for its study since inoculation of pigs has been the only method available. Pig studies are not easily done, and, consequently, our knowledge of hog cholera virus has not kept pace with that available for those viruses that have been cultivated in tissue culture with observable cytopathic effects (CPE).

Hog cholera virus has been propagated in tissue culture(1-10), but either cellular changes were not observed or were not easily recognized, and surety that virus was present required a test in pigs. Other animal viruses, and in particular virus diarrhea virus, also have been observed to behave in this manner; yet, persistent attempts with different strains(11) eventually located some that were cytopathogenic. Also, modifying mediums used for culture of cells proved successful for some viruses(12,13).

This report concerns the culture of a particular strain of hog cholera virus that produces CPE.

Tissue culture method. Primary monolayer cultures were prepared from kidneys of pigs 5 to 16 weeks of age. Growth was initiated with Earle's balanced salt solution (80%), 0.5% lactalbumin hydrolysate (10%), bovine serum (10%), penicillin (500 units per ml), streptomycin (100 mg per ml), and mycostatin (100 units per ml). Excellent cell sheets were produced and were maintained in good condition for at least 2 months by a change of medium twice a week.

At time of virus inoculation, some of the tubes were washed with buffered salt solution and a type of medium used successfully by Karzon for cultivation of distemper virus was substituted(12). This medium consisted of 50% bovine amniotic fluid, 46% Earle's salt solution and either 4% lamb serum, 4% bovine serum, or 4% pig serum. The same antibiotics as used in the original medium were added.

Virus inoculum. The following strains of hog cholera virus were prepared for inoculation: 1. Virulent strain A(14), maintained as fully virulent virus by transfer in swine. A 10% suspension of spleen from an infected pig was used as inoculum. 2. Modified strain A(14), modified in virulence by 17 serial transfers in rabbits. A 10% suspension of spleen from an infected rabbit was used as inoculum. 3. Persistent strain A(15), initially modified in virulence by transfer in rabbits and then reverted to virulence by establishing a persistent viremia in young pigs. A 50% suspension of blood from an infected pig was used as inoculum. 4. Virulent strain I[†] was inoculated as a 50% suspension of blood from infected swine. 5. Virulent strain II[†], furnished as dried material which was reconstituted before inoculation. 6. Virulent strain III,[†] also furnished as dried material which was reconstituted and then inoculated.

All strains were inoculated in 0.2 ml amounts into each of 5 tubes that contained the different media. Uninoculated tubes were retained for comparison. The tubes were incubated at 37°C for 7 to 10 days before final reading.

Presence of virus. Since the objective sought was a strain of virus that produced CPE, tubes were examined daily for evidence of cellular changes. Whether effects were noted or not, fluids were removed from each group of tubes 7 to 10 days after inoculation, pooled, and 0.2 ml were transferred to other tubes. If no changes were noted after 5

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* The authors acknowledge with appreciation support from the Office of Naval Research.

[†] Virulent strain I was kindly supplied by Dr. Donald P. Gustafson, Purdue Univ. and virulent strains II and III by Dr. Howard W. Dunne, Pennsylvania State Univ.

transfers, further attempts were discontinued. If cell changes were noted, transfers were continued. Results are presented in Table I. In later passages, Parker 199 (86%), lamb serum (4%), 0.5% lactalbumin hydrolysate (10%), plus antibiotics was used. This also was tried with 4% bovine serum substituted for lamb serum.

Sera were procured from 14 pigs at time of inoculation with modified hog cholera virus and again 21 days afterward. For inoculation, Strain A virus was used for 4 pigs and another strain was given the others. Since some pig sera were toxic to tissue-cultured cells, each serum was diluted 5-fold and then tested for its capacity to neutralize virus. Equal portions of diluted serum and undiluted tissue culture fluid from infected tubes were mixed, placed in the refrigerator for 2 hours and 0.2 ml inoculated into each of 5 tubes. At the same time, tissue culture fluid was titrated for virus. Also, comparative virus titrations were made in pigs. Fluids from tubes also were cultured for PPLO and other bacteria. None were demonstrated.

Results. Only strain A, that had been established as a persistent viremia in pigs, produced CPE and only in the medium that contained amniotic fluid and lamb serum (Table I). Subsequent passages showed this to be a reproducible effect during 26 serial passages (Fig. 1) in this medium, although similar changes were noted when Parker 199 and 4% lamb serum was used. As in initial tests, when bovine serum was substituted for lamb serum, CPE did not develop.

TABLE I. Cultivation of Hog Cholera Virus in Tissue Culture.

	Medium for cell maintenance					
	Earle's medium + serum of			Karzon's medium + serum of		
Virus strain	Lamb	Bovine	Pig	Lamb	Bovine	Pig
A virulent	—	—	Toxic	—	—	Toxic
A modified	—	—	"	—	—	"
A persistent	—	—	"	+	—	"
Virulent I	—	—	"	—	—	"
" II	—	—	"	—	—	"
" III	—	—	"	—	—	"

— means no CPE; +, CPE found.

Toxic means cells in uninoculated tubes degenerated.

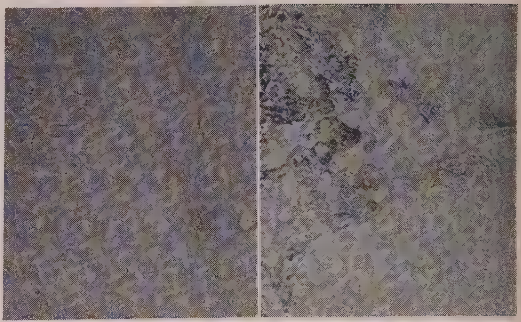

FIG. 1. Uninoculated monolayer of pig kidney cells (left). On right, pig kidney cells infected with hog cholera virus. Note cytopathic effects in the form of cell degeneration and broken cell sheet.

Titrations of tissue culture fluids from inoculated tubes showed approximately 100 $TCID_{50}$. Comparable tests in pigs showed the same endpoints. Tissue-cultured virus from 8, 16 and 32 transfers produced signs of illness and lesions typical of hog cholera. After 3 days temperatures became elevated and the pigs died 9 to 20 days later. Autopsy revealed hemorrhagic lymph nodes and petechiation of kidneys and bladder. Tissue-cultured virus was neutralized by sera from pigs 21 days after inoculation with modified virus but not by sera at time of inoculation.

Discussion. Factors responsible for the CPE produced by this particular strain of hog cholera virus are not shown by these studies. It is important to note that this strain had been modified and reverted from attenuation to virulence in a single pig passage. Furthermore, there were 100 to 1000 times more virus in the inoculum than is ordinarily produced by virulent strains maintained in the usual manner(15). The large amount of virus may have been a factor. Initially, it was thought that the medium was important, and it is, to the extent that lamb serum permitted the virus to be cytopathogenic, while bovine serum did not. Lamb serum, therefore, was an essential constituent whether in combination with amniotic fluid medium or Parker 199. Whatever the reason, a strain of hog cholera virus is now available for study and, since it was neutralizable it should be helpful for serological tests.

Summary. A strain of hog cholera virus has been procured that produced CPE in tissue cultures of pig kidney cells. This tissue-cultured virus produced typical hog cholera when inoculated into susceptible swine and it was neutralized by immune sera.

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Response of Lactic Acid Bacteria to Amino Acid Derivatives. V. L- DL- and D-Valic Acids.* (26216)

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It was recognized as early as 1947(2) that either L- or D-phenyllactic acid, but not D-phenylalanine, is an effective substitute for L-phenylalanine in *Lactobacillus arabinosus* nutrition. Corresponding relationships between the optical forms of phenyllactic acid and phenylalanine were subsequently shown to hold additionally for a variety of other lactic acid bacteria(3). Utilization by *L. arabinosus, Leuconostoc mesenteroides* and *Streptococcus faecalis* of various hydroxy acid analogs in place of the corresponding amino acids was reported by Holden, *et al.* (4), but these authors' data do not include determinations of optical specificity. It was desirable, therefore, to secure more complete information on utilization of optically active hydroxy acids other than phenyllactic acid. The present report concerns utilization of the valine analogs, L-, DL- and D-valic (α-hydroxyisovaleric) acids.

Methods. L- and DL-valic acids were synthesized from L- and DL-valines essentially as described by Baker and Meister(5). D-Valic acid was produced by resolution of DL-valic acid with cinchonine†. Microbiological procedures and medium were the same as those used previously(3) except L-phenylalanine, final concentration 20 mg %, was included in the medium and DL-valine, DL-norleucine and DL-norvaline were omitted. Bacterial cultures have been described(1,6).

Results. Preliminary screening of 10 bacteria revealed that *Lactobacillus arabinosus*

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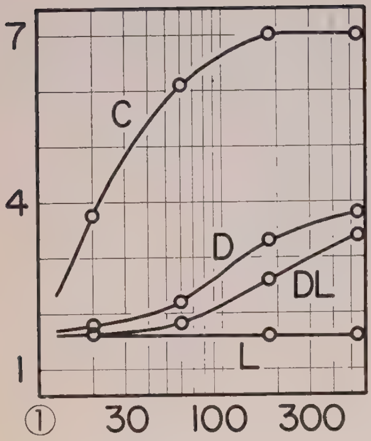
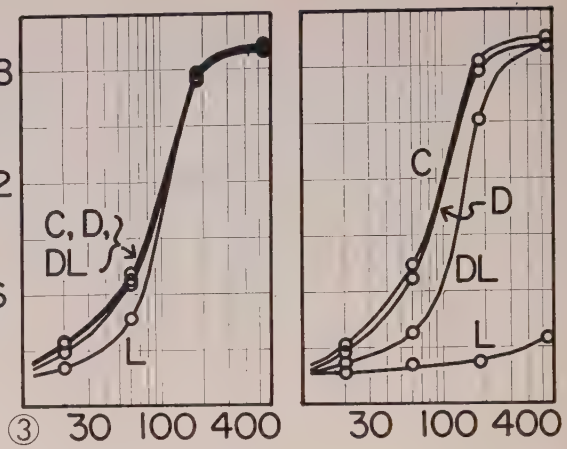
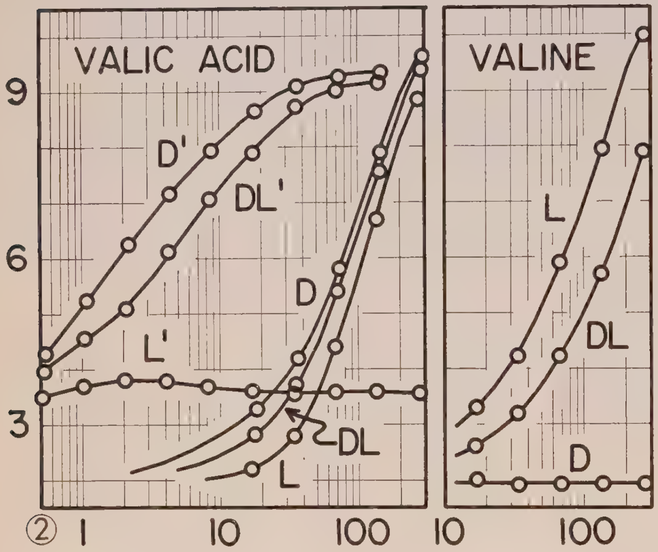
* Paper 133. This work was aided by grants from U.S.P.H.S. and Univ. of California. The authors are indebted to Evelyn Brown and Audree Fowler for assistance. For the preceding paper in this series see Camien and Dunn(1).

† DL-Valic acid 10 g, cinchonine 25 g and dry acetone 50 ml were stirred with a magnetic stirrer for 3 hours in a closed flask. Filtration of mixture yielded 13 g of essentially pure D-valic acid-cinchonine salt. The product was washed with cold acetone to remove residual traces of the highly acetone-soluble L-salt. It is of interest that although this procedure was consistently successful for resolution of DL-valic acid, it appeared to be relatively ineffectual in resolving DL-leucic acid.







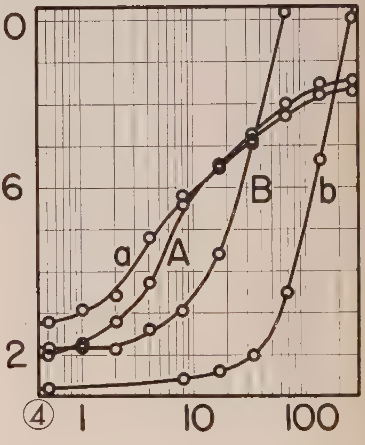


FIG. 1. Responses of *Streptococcus faecalis* 8043 to D-, DL- and L-valic acids (curves D, DL and L, respectively) and to the L-valine control (curve C). Incubation period was 72 hr. Response to L-valic acid remained negligible with incubation periods up to 10 days at test levels up to 4 μM/ml. D-Valic acid under the latter conditions yielded a maximal response nearly equal to that of the L-valine control. Values on the horizontal (logarithmic) scale represent valine and valic acid concentrations in mμM/ml; those on the vertical scale represent ml of 0.01 N sodium hydroxide required to titrate each ml of test culture.

FIG. 2. Responses of *Lactobacillus casei* 280-16A to D-, DL- and L-valic acids (left) and L-, DL- and D-valines (right). Curves D′, DL′ and L′ were obtained when the described medium (see *Methods*) was supplemented with DL-valine; these responses therefore illustrate valic acid utilization as an α-hydroxy acid source rather than as a valine substitute. The remaining curves were obtained in the valine-free test medium supplemented with 1.5 μM of DL-lactic acid (as hydroxy acid source) per ml. The coordinates are on the same bases as in Fig. 1.

FIG. 3. Responses of *Lactobacillus mannitopoeus* to D-, DL- and L-valic acids (curves D, DL and L, respectively) and to the L-valine control (curves C) in the described medium (see *Methods*) with (right-hand figure) and without (left-hand figure) the addition of DL-α-hydroxybutyric acid at 34 μM/ml. The coordinates are on the same bases as in Fig. 1.

FIG. 4. Responses of *Lactobacillus casei* 280-16A to DL-valic acid in the described medium (see *Methods*) supplemented with DL-valine at 20 mg % (curves a, A) and in the same medium containing NZ-Case at 600 mg % in place of all the amino acids (curves b, B). Curves A and B were obtained when Tween 40 at 4 mg % was added to the test media. Curves a and b are the controls (no Tween 40). The coordinates are on the same bases as in Fig. 1.

8014, *Lactobacillus buchneri*,‡ *Lactobacillus casei* 7469, *Lactobacillus fermenti* 9338 and *Lactobacillus mannitopoeus*‡ were able to substitute DL-valic acid for L-valine with little or no loss of efficiency. *Leuconostoc citrovorum* 8081,§ in some experiments but not in others, gave a delayed response to valic acid at the usual levels, suggesting a capacity of this organism to utilize the hydroxy acid through a readily occurring mutation. *Streptococcus faecalis* 8043 required exceptionally high concentrations of valic acid in substitute for L-valine and was unique in its specificity for D-valic acid in this respect (Fig. 1). *Lactobacillus brevis* 8287,† *Lactobacillus gayonii* 8289† and *Lactobacillus lycopersici* 4005† did not utilize DL-valic acid at test levels up to 3.2 μM per ml in place of L-valine.

Optical specificity of valic acid utilization was not marked except for *S. faecalis* as already mentioned and in the special case of *L. casei* 280-16A to be considered further on. D-Valic acid was slightly preferred by *L. buchneri, L. casei* 7469 and *L. mannitopoeus*, whereas L-valic acid was somewhat better for *L. arabinosus* and *L. fermenti*. *L. casei* 280-16A has a special requirement for D-α-hydroxy acids(1) which prompted the following modifications of testing procedure: a) the described medium (see *Methods*) was supplemented with 1.5 μM of DL-lactic acid (as D-α-hydroxy acid source) per ml to permit tests of valic acid as a valine substitute (Fig. 2-left, curves D, DL and L; corresponding valine controls in Fig. 2-right), and b) the lactic acid supplement was omitted and DL-valine was added at 20 mg % to permit tests of valic acid as a D-α-hydroxy acid source (Fig. 2-left, curves D′, DL′ and L′). It is apparent from these data (Fig. 2) that the valine requirement of *L. casei* 280-16A is L-specific, that utilization of valic acid as an hydroxy acid source for this organism is D-specific, and that utilization of valic acid by this strain as a valine substitute is essentially lacking in optical specificity.

DL-α-Hydroxybutyric acid,‖ α-hydroxyisobutyric acid,‖ DL-α-hydroxy-α-methylbutyric acid,‖ DL-α-aminobutyric acid and α-aminoisobutyric acid were screened as potential antagonists of L-valine and DL-valic acid utilization because of their apparent structural similarities to the latter compounds. The most impressive inhibitory effects were produced by DL-α-hydroxybutyric acid, while DL-α-hydroxy-α-methylbutyric acid and DL-α-aminobutyric acid appeared to be similar but less potent inhibitors. α-Hydroxyisobutyric acid did not effectively inhibit any of the test organisms, whereas α-amino isobutyric acid strongly inhibited *L. casei, L. buchneri* and *L. mannitopoeus*, but not *L. arabinosus* and *L. fermenti*. The α-aminoisobutyric acid inhibition of *L. casei* was exceptional in that its reversal was effected by alanine peptides (data to be presented later) but not by either L-valine or DL-valic acid. Data representative of final tests with DL-α-hydroxybutyric acid are shown in Fig. 3. These response curves (Fig. 3) show that the inhibitor at 36 μM/ml suppresses utilization by *L. mannitopoeus* of L-valic acid but not that of either D-valic acid or L-valine. Data analogous to those presented in Fig. 3 were either interpolated or extrapolated to provide the condensed summary of DL-α-hydroxybutyric acid effects listed in Table I. It is apparent from these data (Table I) that L-valine is generally less susceptible than valic acid to antagonism by DL-α-hydroxybutyric acid and that L-valic acid is often markedly less potent than D-valic acid in reversing the effects of this antagonism.

Additions of Tween 40 (Atlas Powder Co. polyoxyethylene sorbitan monopalmitate) to the present experimental medium did not enhance valic acid utilization, although marked increases in growth-promoting potencies of various α-hydroxy acids for *L. casei* 280-16 had previously been observed in media supplemented with this non-ionic detergent(8). The apparent discrepancy was traced to a

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‡ Three-fourths of the glucose was replaced with separately sterilized L-arabinose in media for the indicated organisms, for which a pentose supplement is highly stimulatory(7).

§ The medium for this organism was supplemented with 15 mμg of calcium leucovorin per ml.

‖ Test solutions were adjusted to pH 6.5 with sodium hydroxide.

TABLE I. Effects of DL-α-Hydroxybutyric Acid on Responses of Five Lactic Acid Bacteria to L-Valine and L-, DL- and D-Valic Acids.

Organism	DL-α-Hydroxybutyric acid conc. (μM/ml)	Substrate concentration required to yield half maximal response			
		L-valine	Valic acid L-	DL-	D-
		(mμM/ml)			
L. casei 7469	0	49	79	62	55
	4	54	116	68	60
	12	54	436	96	82
	36	57	470	180	158
	108	60	1600	690	690
L. arabinosus	0	48	54	81	85
	4	47	62	93	120
	12	48	76	106	139
	36	46	115	160	166
	108	53	240	250	290
L. buchneri	0	134	220	174	148
	4	125	1000	167	134
	12	137	2600	240	148
	36	140	>3000	350	200
	108	166	>3000	630	380
L. mannitopoeus	0	76	94	84	82
	4	84	570	94	75
	12	84	1600	116	88
	36	88	>3000	230	88
	108	83	>3000	400	186
L. fermenti	0	89	104	116	171
	4	89	145	145	184
	12	71	190	152	200
	36	75	280	290	320
	108	88	840	540	440

marked inhibition of α-hydroxy acid utilization by enzymatically digested casein, which was included in earlier media(8). Antagonism of this inhibition is readily effected by Tween 40, which thus appears to potentiate the growth-promoting properties of α-hydroxy acids in media containing enzymatically digested casein, but not in media lacking such an inhibitor. These relationships are illustrated (Fig. 4) by the effects of Tween 40 on response of *L. casei* 280-16A to DL-valic acid in the present test medium (supplemented with DL-valine) and in a medium containing NZ-Case (Sheffield Chemical Company's enzymatic digest of casein). Further data concerning inhibition of α-hydroxy acid utilization by peptide derivatives from casein and other proteins are to be presented later.

Discussion. The present findings that whereas valine utilization is markedly L-specific, utilization of the valine substitute, valic acid, is either D-specific or relatively lacking in optical specificity closely parallel those of earlier investigations(2,3) of phenylalanine and its nutritional substitute, phenyllactic acid. In either case it seems probable that the hydroxy acid is utilized through conversion to the corresponding amino acid essentially as proposed by Holden *et al.*(4), *i.e. via* dehydrogenation to the α-keto acid and subsequent transamination. Lack of optical specificity toward the α-hydroxy acids when these are used in place of the corresponding α-amino acids suggests that some of the organisms may possess appropriate α-hydroxy acid racemases, but this does not appear to be true of *L. casei* 280-16A, which is evidently incapable of racemizing either L-valic acid (curve L′, Fig. 2) or phenyllactic acid (1). It seems likely, therefore, that *L. casei* 280-16A possesses separate L-specific and D-specific dehydrogenases which account for utilization of either optical form of these hydroxy acids in place of the corresponding L-amino acids. Optically selective antagonisms of hydroxy acid utilization (Table I) are of considerable interest, but since various metabolic steps (*e.g.* cell penetration, racemization, dehydrogenation) might be involved in such antagonisms, these results are not unequivocally informative.

Summary. Substitution of L-, DL- and D-valic acids in place of valine in the nutrition of 10 lactic acid bacteria has been investigated. Five of the bacteria were able to utilize either optical form of valic acid efficiently in place of L-valine, even though the requirement for this amino acid is highly L-specific. Three of the latter 5 bacteria, on the other hand, could be made to exhibit a marked preference for D-valic acid by selective antagonism of L-valic acid with the inhibitor, DL-α-hydroxybutyric acid. One other organism showed a similar preference for D-valic acid without use of the inhibitor, but in this case utilization of D-valic acid was relatively inefficient. The α-hydroxy acid-dependent organism, *L. casei* 280-16A, was D-specific in its utilization of valic acid as an α-hydroxy acid source, but was not optically specific in its utilization of valic acid as a valine substitute.

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